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Comparative Biochemical Studies on Venoms of Snakes of Costa Rica.

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ABSTRACT

Scientists have depended largely upon morphological characteristics to identify organisms, estimate their relationships, and follow their evolution. As morphological variation is the result of underlying biochemical differences, comparisons of biochemical similarities and differences should give more sensitive indications of natural relationships than gross morphology.

Venom constituents of seven species of Bothrops, two of Crotalus, Lachesis muta, Micrurus nigrocinctus and Naja naja were compared at the intraspecific, specific, generic and family levels. Venom proteins of each species were fractionated by starch-gel electrophoresis in borate buffer of pH 8.6. Enzyme activities of B. atrox, B. nummifera and C. durissus venoms were assayed, localized on electropherograms and checked for toxicity.

Venoms of Crotalidae contain potent enzymes with trypsin, chymotrypsin, rennin and papain-like activities. Fibrinolysin, migrating with proteases, is present in all venoms; thrombin usually is present. Proteolytic and

thrombic activities are the most toxic electrophoretic fractions. The anodal migration of highly concentrated L-amino acid dehydrogenases is faster in Bothrops and Lachesis than in Crotalus. Phosphatidases, rapid anodal fractions, are very active in B. atrox, B. schlegeli and C. atrox, and weak in B. nummifera, B. picadoi, B. nasuta and C. durissus. Phosphatases of slow migration and hyaluronidase are present in all venoms. Protein electropherograms show a predominance of anodal fractions. The 25% solid of the venom is three fourths protein. Sodium is usually the predominate cation, chloride is low and phosphate is absent; pH averages 5.8. Osmotic pressure, averaging 340 mOsm/L, approximates that of snake blood.

Acetylcholinesterase usually is present in elapid venoms but is not the cause of their strong neurotoxicity as M. nigrocinctus venom lacks the enzyme. Potent direct and indirect hemolysins are present. Electrophoretic fractions are predominately cathodal.

Developmental factors must be considered in evaluating the taxonomic significance of components of snake venoms. Newborn C. durissus and B. nummifera lack L-amino acid dehydrogenase and other venom enzymes which are present in older snakes. Physiological effects may also be important

as venom of certain adult jumping vipers also lack L-amino acid dehydrogenase.

Certain individuals of single populations from restricted geographic areas could be identified by differences in number, migration rate and quantity of proteins fractionated by electrophoresis. In one population of fer-de-lance electropherograms of eight individuals were all different. Although no single venom protein was restricted to a specific population, the frequency of occurrence of polymorphic forms of L-amino acid dehydrogenase and tryptic-like activity was different in populations from the Atlantic and Pacific sides of the continental divide. Such variations were not correlated with size, duration of captivity, feeding, time of milking or duration of venom storage.

Populations of B. nummifera captured in the Atlantic drainage could be distinguished from populations from the Pacific drainage by their proteolytic enzymes. Proteolytic activity migrated in a single band in all individuals but was localized in a cathodal band in Pacific populations and in an anodal band in Atlantic populations. Although critical intergrades have not been found, the two forms should be classified within the same species. Except for proteolytic fractions, the electropherograms of venoms from

the two areas were identical. Venoms from both forms contained high potassium and low sodium concentrations. This is unique among Bothrops studied.

Although intraspecific variations occurred, each species could be identified from more uniform and generalized characteristics of their electrophoretic patterns. Such patterns clearly differentiated B. picadoi from B. nummifera, two species difficult to distinguish morphologically. C. durissus contained both coagulant and anticoagulant activities; C. atrox possessed only anticoagulant activity.

Considering the great individual and regional variability of snake venom proteins, venom from individual snakes rather than pooled samples should be utilized more often in research.

INTRODUCTION

Snakebite is a serious medical problem in some areas of the world. While less than 15 deaths are reported annually in Europe due to this cause, 7,000 to 12,000 deaths occur in India each year (Porges, 1953; Minton, 1957). At least 1,500 persons a year are bitten by venomous reptiles in the United States. Between 1950 and 1954, 215 deaths from venomous animals, 71 from poisonous reptiles, were reported in the United States (Parish, 1959; Russell, 1961). It is estimated that 35,000 accidents by venomous snakes occur each year in Central and South America. This is partly due to the abundance of snakes in tropical America. For example, 180,000 rattlesnakes and 290,000 specimens of Bothrops jararaca were collected in southern Brazil and examined at the Butantan Institute in Sao Paulo, Brazil, during its first 60 years of existence (Bücherl, 1961). Even though most farmers in areas densely populated by venomous snakes keep a stock of antivenom, in 1962 thirty-two persons died from snakebite in Costa Rica, a country of a million and a quarter inhabitants. This figure represents 0.3% of the mortality of that country (Mekbel, personal communication).

Venomous Animals

Venomous secretions are produced by many animals other than snakes. They represent many of the phyla of the animal kingdom (Phisalix, 1922; Porges, 1953; Halstead, 1956, 1959). Sponges and coelenterates produce toxins that cause skin irritation or even kill. Octupuses possess well-developed venom organs; fatalities have been reported from their venoms. Some marine annelids possess stinging setae; other species have chitinous jaws capable of inflicting venomous bites. Some of the many venomous arthropods, e.g. spiders and myriapods, have venom glands located in their mouths; in others, e.g. scorpions, wasps and bees, the venom gland and stinging organ are in the hinder parts of their bodies. Venom glands are distributed throughout the skin of frogs and salamanders. Venomous fish are found among elasmobranchs and teleosts (Russell and Lewis, 1956). The Gila monster and its relative the Mexican beaded lizard are the only venomous lizards (Tinkham, 1956). Only two venomous mammals are known: the male duck-billed platypus of Australia and the short-tailed shrew of America (Pearson, 1956).

Venomous Snakes

Of venomous animals, snakes are undoubtedly the greatest problem to man. Of the 2,500 species in the world, 600 possess venom glands, but only 150 species are dangerous. Poisonous species occur in six families; these have been

placed into four groups on the basis of tooth structure (Ditmars, 1946).

AGLYPHA: solid teeth; most species non-poisonous.

Amblycephalidae. Saliva poisonous but no venom-injecting apparatus.

OPISTHOGLYPHA: pair of grooved teeth in rear of upper jaw (rear-fanged snakes); considered mildly poisonous.

Colubridae. Inefficient venom apparatus.

PROTEROGLYPHA: two front teeth of upper jaw modified into rigidly set, grooved fangs; very poisonous.

Hydrophydae. Primitive injecting apparatus (e.g. sea snakes, Pelamis).

Elapidae. More advanced injecting apparatus (e.g. king cobra, Naja; coral, Micrurus).

SOLENOGLYPHA: two front teeth of upper jaw modified into long, movable, hollow fangs that can be folded against the roof of the jaw (viperine snakes). Highly advanced injecting apparatus; very poisonous.

Viperidae. True vipers.

Crotalidae. Pit vipers (e.g. fer-de-lance, Bothrops; rattlesnakes, Crotalus; bushmaster, Lachesis).

Snakes are most common in tropical or subtropical climates, but a few species of viper are present as far

north as Scandinavia and Siberia. All poisonous snakes of Europe are true vipers. America lacks snakes of the Viperidae but has Crotalidae, Elapidae and Hydrophydae. Most poisonous snakes of the New World are crotalids. Rattlesnakes (Genus Crotalus) are the characteristic pit viper type of North America, whereas the lance-head snakes of genus Bothrops have their headquarters in tropical America. Africa has no pit vipers. Australian poisonous snakes are limited to Elapidae. All families are found in Asia. There are no land-dwelling venomous snakes in Cuba, Jamaica, Haiti and Dominican Republic, Puerto Rico, Iceland, Ireland, Chile, New Zealand, Madagascar and the Polynesian, Canary and Azores Islands. This is a remarkable feature if we consider the luxuriant flora and the proper climate for reptile development in some of those countries and their proximity to territories which do have large populations of poisonous species (Ditmars, 1946; Pollard, 1956).

Ancient Studies on Snake Venoms

Since antiquity, mankind has worried about the bites and stings of venomous animals. There is no doubt that snakes have played a very important role in history. Serpents represent not only the temptation of original sin, but also wisdom and skill. They have been associated with the medical sciences since ancient times. The modern symbol of medicine, the caduceus, is a staff intertwined with serpents.

Even the oldest medical writings, the ancient Egyptian papyri, circa 1600 B.C., included prescriptions to be used against diverse bites. Greeks and Latins took over this empirical medical knowledge. Matthioli's revision of Dioscorides De Materia Medica, printed in Venice in 1554, contains many references to studies on venoms of scorpions, snakes and shrew. Leake (1956) states that twenty-five sections of Book V of the Seven Books of Paulus Aeginata, the best summary of classical Greco-Roman and Arabic medicine, deal with venomous animals and indications for treatment of persons bitten or stung. Ancient knowledge of venomology was transmitted to medieval and Renaissance Europe through Byzantine and Arabic writings. In The Workes of that Famous Chirurgion Ambrose Parey, an English translation of a book written by the French surgeon Ambroise Paré (London, 1634), there is an illustrated and detailed discussion of poisonous animals. A contemporary of Paré, the Parisian Jacques Grevin is credited with the first systematic account of poisons, Deux livres des venins anvers (Antwerp, 1568). One volume deals with venomous animals and includes illustrations to identify the various snakes and other poisonous animals known at that time.

Modern Studies on Snake Venoms

It was not until the seventeenth century that the first methodical work on snake poisons was done by Francesco Redi (Osservazioni in torno alle vipere, 1664). He demonstrated

that in order for snake venoms to produce their effects, they had to be injected under the skin, whereas they were harmless when taken by mouth. Felice Fontana extended these studies to many other poisonous materials. In his study of snake venoms, Richerche fichiche sopra il deleno della vipera, published in 1767 and considered as the starting point for modern systematic work on venoms and toxicology, he described snake venoms as blood coagulants and anti-coagulants (Klobusitzky, 1961).

The studies of Redi and Fontana were not followed by any scientific work on venoms for almost another century. In the latter part of the nineteenth century, Silas Wier Mitchell, a great Philadelphia neurologist, published significant studies on rattlesnake venom and other serpent venoms (Researches upon the venom of the rattlesnake, Smithsonian Institution, Washington, D.C., 1860). The same investigator, together with Reichert, published Researches upon the venoms of poisonous serpents in 1886. They showed for the first time that snake venoms are proteinaceous and have characteristic toxic effects on nerve tissue and on blood. By that time, taxonomy had developed sufficiently to give a reasonable classification of poisonous animals. The first systematic description of venomous snakes was made by Fayrer in 1872.

Calmette, a distinguished bacteriologist and immunologist, initiated studies on immunization against snake venoms and published in 1896 a small volume on this subject.

Pasteur Institutes throughout the world undertook the supply of antivenoms (Calmette, 1894, 1895, 1898). Vital Brazil, founder of the Butantan Institute of Serum Therapy of São Paulo, Brazil, also made valuable studies on immunization and treatment of snake bite. He wrote A Defensa contra o Ophidismo in 1911. Detailed studies on the hemolytic properties and other effects of snake venoms on blood were performed by Flexner and Noguchi in 1902 in the United States. A book entitled Snake Venoms was written by Noguchi in 1909. About that time Kyes in Germany, studied cobra venom and showed that lecithin was necessary for the action of cobra hemolysin. Two volumes entitled Animaux Venimeux et Venins, with a total of 1,520 pages, were written by Marie Phisalix (1922). All knowledge on venoms up to 1922 was compiled in these volumes.

Pharmacological Classification of Snake Venoms

Venoms have been long classified into two types on the basis of whether their toxic action is local or generalized. Those that possess a predominant local action are commonly called the bothropic type, as venoms of many species of the genus Bothrops exhibit such action. Venoms from North American rattlesnakes and true vipers also belong to this group. Venoms that possess a powerful systemic action are commonly called the elapid type, as venom from many elapid snakes exhibits such action. They are also termed neurotoxic, since substances acting on the nervous system are credited

with their extremely high lethality. Such a classification is not absolute, since venom of some species exhibits both strong local and systemic effects (Picado, 1931; Van Heyningen, 1954).

Chemical Composition of Snake Venoms

Proteins are the toxic substances present in snake venoms. According to a review of animal poisons by Kellaway (1939), toxic proteins may be classified in three types: proteolytic enzymes, hemolysins and neurotoxins. The proteolytic activity of venoms was recognized by Lacerda in bothropic venoms since 1881. This was the first discovery of an enzyme in animal poisons, but the possibility that reptile venoms might contain agents similar to those of the digestive juices was suggested even earlier, according to Zeller (1951). Three main effects have been attributed to snake venom proteases: (1) hemorrhagic and destructive effects at the site of the bite and in the viscera; (2) coagulation or anticoagulation of blood; and (3) hypotensive action due to damage to vascular endothelium with subsequent escape of blood from the vessels and to the liberation of histamine-like substances. (Van Heyningen, 1954; Zuber, et al., 1960).

Some coagulant venoms possess thrombin-like properties, since they are able to coagulate pure fibrinogen in vitro without calcium ions, tissue extracts or prothrombin. Other venoms exert their promoting action on clotting by convert-

ing prothrombin to thrombin. Anticoagulant activity is also found in some venoms. The discovery that coagulant and anticoagulant substances may coexist in the same venom dates back to 1886. Much controversy has existed around the multiple and apparently antagonistic actions of venoms on the blood clotting process and on the relationship of coagulant and anticoagulant properties to proteolytic activity. An excellent review of all papers published on the action of snake venoms on blood clotting was made by Klobusitzky (1961).

Hemolytic properties of venoms were found to be due to the presence of phosphatidase A, an indirect hemolysin, which converts lecithin into lysolecithin, a substance that ruptures red blood cells. Some venoms show direct hemolytic action not dependent on the presence of lecithin. The role of phosphatidase A in snake venom poisoning probably consists of rupture of erythrocytes and injury to capillary endothelium, leading to hemorrhage, edema and necrosis (Porges, 1953; Van Heyningen, 1954).

Neurotoxins, characteristic components of venoms of cobras and allied species of family Elapidae, seem to act selectively on the peripheral nervous system, thus inhibiting respiratory movements and producing death by asphyxia (Porges, 1953; Van Heyningen, 1954).

Hyaluronidase was discovered in snake venoms by Duran Reynals in 1936. This enzyme is known as the "spreading factor" because it enhances the spreading of infectious

agents and dyestuffs in the skin of animals, and was found in cultures of invasive bacteria, in mammalian testes, hookworms and leech heads. It was further discovered in venoms of bees, scorpions and poisonous fish. It hydrolyses hyaluronic acid and thus allows a rapid diffusion of toxic substances and foreign matter by reducing the viscosity and resistance of the ground substance (Van Heyningen, 1954; Rogers, 1961).

Indian investigators found acetylcholinesterase in cobra venom (Iyengar, et al., 1938). It was one hundred times more active than the esterase of the electric organs of the electric eel, the most active source of the enzyme known until that time. Further studies demonstrated its presence in all elapid venoms and absence in the viperine venoms (Zeller, 1951). This enzyme was suspected of being responsible for the neurotoxic properties of cobra venom, since its interference with acetylcholine output of nerve endings might result in a curare-like effect on cholinergic motor nerves. However, very conclusive experiments have demonstrated that the neurotoxin of elapid venoms is a substance different from true acetylcholinesterase (Detrait, et al., 1959).

An unspecific phosphodiesterase able to release phenol, but not phosphoric acid from diphenyl phosphate esters was the first phosphatase found in snake venoms. Additional specific phosphatases have since been found in these secretions. Ribonuclease and deoxyribonuclease are believed to

supplement the hydrolytic activity of other enzymes and to contribute to the cytolytic effects of venoms (Taborda, et al., 1952a; Porges, 1953). Nicotinamide-dinucleotidase (diphosphopyridine-nucleotidase) catalyzes the hydrolysis of NAD^+, thus interfering indirectly with tissue oxidation. Adenosine-triphosphatase probably produces shock by exhausting supplies of the readily available energy of adenosine-triphosphate. Adenosine-5'-monophosphatase or 5-nucleotidase seems to be present in all snake venoms and is usually the most active phosphatase (Zeller, 1951; Van Heyningen, 1954).

A nonhydrolytic enzyme, L-amino acid oxidase, was demonstrated for the first time in snake venoms by Zeller and Maritz (1944). Its coenzyme, flavin adenine dinucleotide is the substance to which the yellow color of most snake venoms is due. This enzyme, often called ophio-oxidase, has been isolated in a pure state from moccasin venom and is about 500 times as active as mammalian L-amino acid oxidases. Although considered unnecessary for the lethal effects of venoms, it is credited with a protease-enhancing effect, destroying amino-acids resulting from proteolysis (Porges, 1953). Venoms appeared as more complex secretions than the ordinary digestive juices, which do not contain any hyaluronidase or L-amino oxidase.

Zeller (1951) reviewed studies on snake venoms, particularly papers published during the previous decade. He believed that the potent biological catalysts are not accidental components of snake venoms. The role of enzymes

in the mechanism of poisoning is not clear and some investigators have demonstrated that toxicity of some snake venoms is not associated with any known enzymatic action (Yang, et al., 1959; Detrait, et al., 1959). On the other hand, some crotalid venoms are very potent coagulants due to thrombin-like substances (Eagle, 1937) and the toxicity of thrombin has been demonstrated (Marciniak, et al., 1962).

Venoms of Other Animals

Venoms of other animals show some similarities to snake venoms. Toxins of spider venoms are proteinaceous (Kaiser and Michl, 1958). Trypsin, hyaluronidase and lecithinase activities have been detected in venoms of some spiders (Kaiser, 1956; Russell, 1961). Venom of spiders of genus Phoneutria is very similar to elapid venom in its neurotoxic properties. Yet, poison of other spiders (Lycosa) resembles crotalid venoms in its action (Van Heyningen, 1954; Bücherl, 1956). Scorpion venoms are more toxic than spider venoms and very similar in action to those produced by elapids (Del Pozo, 1956). Histamine-, acetylcholine- and bradikinin-like actions have been demonstrated in venoms of Brazilian scorpions (Diniz and Gonçalves, 1956). Venoms of North African scorpions show lecithinase but no proteolytic activity (Balozet, 1956). Stingray venom is also of protein nature, very labile and easy to inactivate by heating. It is less toxic than rattlesnake and black widow spider venoms (Russell, 1961). Neurotoxins and cytotoxins

seem to be present in venom of the Gila monster (Tinkham, 1956). Almost nothing is known of the chemical nature and pharmacological actions of venom produced by the submaxillary glands of the American short-tailed shrew (Pearson, 1956). Proteins, basic polypeptides, amino acids and 5-hydroxy-tryptamine are present in skin venomous secretions of amphibians; the peptide fraction exhibits hemolytic properties. Skin secretion and venom produced by parotid glands of toads are different from other amphibian venoms in having steroids with digitalis-like action and phenolic amines which raise blood pressure (Jensen and Westphal, 1956; Kiss and Michl, 1962).

Fractionation of Venom Constituents

The efforts of biochemists working with snake venoms have concentrated for many years on the separation of the above mentioned activities. The goal of such works has been to correlate toxicity with a specific biochemical activity or entity.

In 1938, Slotta and Fraenkel-Conrat obtained by fractional precipitation a crystalline protein, crotoxin, that possessed both neurotoxic and hemolytic properties, from the venom of the Brazilian rattlesnake, Crotalus durissus terrificus. It accounted for most of the toxicity of the venom and they assumed it was a pure substance. (Slotta and Fraenkel-Conrat, 1938, 1939; Slotta, 1956). Indian biochemists suggested that crotoxin was not a pure protein,

but was a mixture of neurotoxic and hemolytic components. They were able, by using similar procedures, to isolate a relatively non-neurotoxic lecithinase from cobra venom (Ghosh and De, 1937).

Chromatographic methods represent a further step in the fractionation study of venoms. Extensive work has been done by venom biochemists, especially in connection with phosphodiesterase (Sinsheimer and Koerner, 1952; Privat de Garilhe and Laskowsky, 1955; Boman, 1959). This enzyme was isolated from the venom of the fer-de-lance and the eastern diamondback rattlesnake by successive chromatography on carboxymethylcellulose, followed by ion exchange columns (Felix, et al., 1960). Column chromatography has also been used to fractionate venoms of Vipera palestinae (Kochwa, et al., 1960) and of Formosan and other Asiatic snakes (Yang, et al., 1958; Ohsaka, 1960). Paper chromatography was employed by Yugoslavian workers to separate venom proteins of Vipera ammodytes (Piantanida and Muić, 1953).

Gel filtration has also been applied to fractionation studies of snake venoms. This is a recent technique based on the fact that substances of large molecular size tend to move at a faster rate than those of small size when a mixture is filtered through a column of ground dextran gel. It allows not only to separate amino acids, peptides and proteins, but also to concentrate biological materials (Porath, 1959; Porath and Flodin, 1959; Flodin, et al., 1960). It was applied by Björk and Porath (1959) to

fractionate ringhals cobra and eastern diamondback rattlesnake venoms. They demonstrated that lecithinase A was the only enzyme retarded to any appreciable degree, in agreement with previous work that had demonstrated a rather low molecular weight for that substance. Ultrafiltration through collodion and cellophane membranes has been employed by Bussard (1955) to demonstrate that a neurotoxic substance of cobra (N. tripudians) venom is of relatively small molecular size.

Although moving boundary electrophoresis has been employed in venom work (Poulson, et al., 1946; Grasset, et al., 1956), zone electrophoresis is the technique that has played an outstanding role. It differs from the moving boundary technique in that electromigration of charged particles occurs through conducting solutions previously stabilized with agar, gelatin, starch, filter paper, etc. It is particularly useful because it allows an absolute separation and identification of very small amounts of biological material (McDonald, 1955). Paper electrophoresis has been the preferred procedure by most venom biochemists for many years. In fact, the technique originated in connection with venom research when Klobusitzky and König, working in Brazil in 1938, put one drop of diluted venom of Bothrops jararaca on a short strip of paper moistened with a solution of a diluted electrolyte and observed that the yellow spot of venom migrated toward the anode upon allowing the current to flow (Klobusitzky and König, 1939).

Grassman and Hannig (1954) fractionated and made a comparative study of snake and insect venoms by means of paper electrophoresis. Michl (1954) applied the same technique to study Bothrops jararaca venom and localized the enzymatic activities along the paper strip. Grasset and Schwartz (1955) demonstrated that substances showing coagulant and anticoagulant properties and simultaneously present in Vipera russelli venom could be separated in bands of different migration by means of paper electrophoresis.

Ohsaka, et al. (1960) employed zone electrophoresis through columns of potato starch to fractionate Trimeresurus flavoviridis venom and identified two hemorrhagic principles of different electrophoretic mobility. Yang, et al. (1959a, b) used the same technique to study the toxicity of enzymes of Formosan cobra venom and other venoms. By means of electrophoresis through agar, Detrait, et al. (1959) were able to separate the neurotoxin of venoms from two species of cobras and demonstrate that the toxic fractions contained no enzymatic action, whereas most of other fractions showed cholinesterase activity.

By employing starch-gel electrophoresis, a recently developed technique of great resolving power (Smithies, 1955, 1959a, 1959b), Jiménez-Porras (1961) obtained 9 fractions and localized 10 enzymatic activities along the electrophoretic pattern of Crotalus atrox venom. He detected phosphatidase A activity in two separate fast migrating anodal bands.

Some investigators have combined several techniques to isolate, purify and identify venom enzymes. For instance, Björk (1961) employed anion- and cation-exchange chromatography, gel filtration and calcium phosphate chromatography to purify phosphodiesterase, 5-nucleotidase, lecithinase A and cholinesterase from cobra venom. Acid treatment followed by fractionation with acetone and paper electrophoresis were used by Habermann (1958) to purify the blood clotting enzyme of the venom of Bothrops jararaca. Grasset, et al. (1956) employed moving boundary and paper electrophoresis and immunochemical techniques to compare components of several viper, pit viper and elapid venoms.

Up to the present time, only two substances have been isolated in crystalline form from nearly 400 snake venoms that have been studied. One of them is crotoxin and the other is the lecithinase of cobra venom. Since the latter is relatively non-toxic, crotoxin remains as the only crystalline toxin isolated from venoms (Slotta, 1956; Klobusitzky, 1961).

Importance of Research on Venoms

As can be inferred from the above review, the subject of venoms is a very complex field which offers research problems in many phases of science. Work with snake venoms is necessary to obtain a clearer understanding of symptoms and treatment of snake bite. Is the toxicity of snake venom due to a specific protein or to a synergistic effect of the

battery of proteins of the venom? If toxicity is due to only one or a few substances, these should be utilized as antigens in preparing antisera to be used in treatment. But venom fractions themselves have medical importance since some of them are useful drugs: cobra neurotoxin is a potent analgesic that possesses several advantages over morphine (Macht, 1941). Snake venom preparations are the strongest hemostatic agents known and are valuable for treatment of coagulation disorders (Klobusitzky, 1959; Kürn, 1959).

Even to the biochemist not interested in toxicological problems, some snake venom enzymes are excellent tools in structural and preparative studies. This point is illustrated by the importance of venom phosphodiesterase in unravelling the structure of nucleic acids (Privat de Garilhe and Laskowsky, 1955; Boman, 1959; Razzell and Khorana, 1959a, b; Felix, et al., 1960). Similarly, venom L-amino acid oxidase has been used to prepare α-ketoacids used in studies of intermediary metabolism (Meister, 1956).

A most recent trend in the field of venomology is the use of venom properties as tools for taxonomic and phylogenetic studies. Minton (1956) found some phylogenetic relationships among pit vipers (Sistrurus, Crotalus, Agkistrodon) on the basis of biochemical and toxicological properties of their venoms. By means of paper electrophoresis, Gonçalves and Vieira studied rattlesnake venom from specimens of Crotalus durissus terrificus collected in northern, central and southern Brazil and found that a

protein of cathodal nature and neurotoxic properties, which they called crotamine, was present only in venom of specimens from southern Brazil (Gonçalves and Vieira, 1950; Gonçalves, 1951). They also observed that southern specimens produced white venom whereas the northern crotamine-lacking venoms were yellow. Further studies by means of ultracentrifugation, paper electrophoresis, antigen-antibody reaction in agar gel and toxicity tests have clearly distinguished between both sorts of venoms. These biochemical findings induced Gonçalves and co-workers to suggest the use of biochemical differences, already extensively utilized with bacteria, for taxonomic purposes at higher phylogenetic levels (Gonçalves and Deutsch, 1956; Gonçalves, 1956). Gonçalves and Vieira (1956) also recognized the crotamine-secreting specimens as belonging to a subspecies which they denominated Crotalus terrificus crotaminicus. Besides their taxonomic implications, such findings have medical importance, since antisera prepared with venom from rattlesnakes from northern Brazil do not prevent death of animals bitten by snakes from southern Brazil. Accordingly, Brazilian workers have emphasized the necessity of preparing regional types of antisera (Gonçalves and Vieira, 1950).

The importance of the study of snake venoms is reflected in the following developments:

(1) One of the major departments of the Butantan

Institute of Serum Therapy of São Paulo, Brazil, has special buildings, research staff and snake parks for the study of the biology of the South American poisonous snakes as well as the physiological, biochemical and pharmacological properties of their venoms and the preparation of antivenoms. This department has brought international fame to the institution, since much valuable research has been done in its laboratories (Ditmars, 1946; Picado, 1931; Víquez, 1935; Bücherl, 1961). To supply the demand for venom, 73,000 snakes were milked at Ross Allen's Reptile Institute of Silver Springs, Florida, during World War II. This institute processed the venom for the United States Armed Forces during that time (Pollard, 1956).

(2) Porges (1953) estimated that more than 6,000 papers were published on the subject of venoms until 1952. The interest in this field has increased since that time. From 1946 to 1954, more than 200 publications appeared concerning research on medical aspects of venoms (Pollard, 1956). A book entitled Die Biochemie der tierischen Gifte was written by Erich Kaiser and Heribert Michl (1958) to review the biochemical research on animal venoms.

(3) An International Conference on Venoms was held in 1954 at the annual meeting of the American Association for the Advancement of Science, in Berkely, California. Sixty-two papers were presented at that meeting by investigators from nineteen countries. A publication entitled VENOMS

(1956), and containing these papers, appeared in 1956.

(4) An International Society of Toxinology was founded in 1961. The first issue of TOXICON, the official journal of the Society, appeared in October, 1962.

Interest and Purposes of Dissertation

My interest in snake venoms began in 1956, when some plant extracts employed by natives to treat venom intoxication were sent to the laboratory where I worked. In the following years, I became aware of the medical problem of snake bite in my country, Costa Rica. Reading on the subject of venoms during my graduate studies in Biochemistry at Louisiana State University, convinced me that medical and toxicological aspects of venomology were only part of the breadth of the field.

I decided to compare venoms of Costa Rican snakes on the basis of their composition and enzyme activities. With the exception of some work performed by Picado (1931) and Víquez (1935) on the biology of Costa Rican venomous snakes and on the toxicological and immunological properties of their venoms, the biochemistry of these secretions remained untouched. Most of our knowledge of properties of venoms of coral snakes, sea snakes and the famous bushmaster, species abundant in Costa Rica, is derived from observations of rare cases of snakebite (Reid, 1956; Halstead, 1959).

For my Master's degree, I had studied the venom of the western diamondback rattlesnake, Crotalus atrox (Jiménez-Porras, 1961) and I decided to compare the venom of that species with C. durissus of Costa Rica. I was particularly interested in comparing actions of the venoms on blood clotting. Coagulant activity is absent in C. atrox, but is present in C. durissus venom. Likewise C. atrox venom exhibits great hemolytic activity, but the venom of the Costa Rican rattlesnake had been reported as lacking hemolysins (Picado, 1931).

Electrophoretic fractionations of venoms were planned for the purposes of determining the toxicity of different proteins, and for comparing similarities and variations in proteins of animals at several taxonomic levels. Results obtained by Gonçalves and co-workers on the influence of geographic distribution on the composition of venom, stimulated the candidate to compare venom proteins of individuals of a species from the same population and from populations of different geographic areas.

Almost all investigators to date have employed dried samples of venoms for their research (Jiménez-Porras, 1961). In order to prevent any alteration in venom proteins, this work was performed with fresh material or samples kept frozen since the animals were milked.

GEOGRAPHIC CONSIDERATIONS AND SOURCE OF ANIMALS

Costa Rica is bounded by Nicaragua, Panama and the Atlantic and Pacific oceans. Its area, 19,238 square miles, is about the size of West Virginia. A mountain chain begins near the Nicaraguan border and extends across the center of the country to and across the Panamanian border (Fig. 1 B). The northern and central part of these mountains is called the Cordillera Volcanica. Nine volcanoes, two active at present, occur along its length. The northern section of the Cordillera Volcanica maintains an average elevation of about 3,300 feet. A pass in the region of Tilaran, where the elevation drops to around 1,500 feet, divides the northern section from the southern section. The southern section has an elevation above 5,000 feet with volcanic peaks as high as 9,100 and 11,300 feet. The central plateau, where the largest cities are found, lies between the Cordillera Volcanica and the southern-most chain of mountains, the Cordillera de Talamanca. These mountains of intrusive origin rise above an average elevation of 6,000 feet to peaks ranging from 10,200 to 12,700 feet. From both sides of the central mountain ridge, lowlands extend to the oceans.

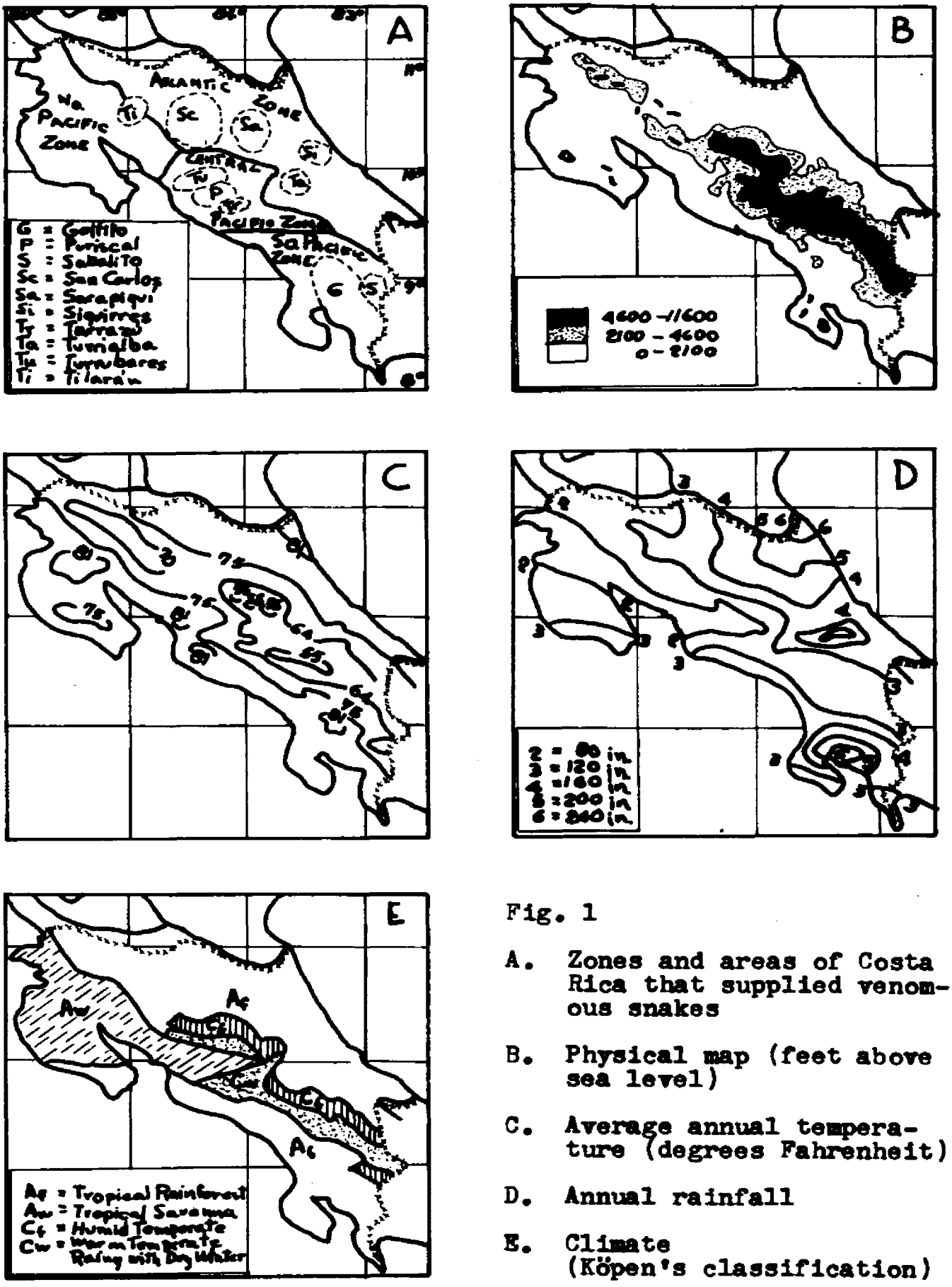


Fig. 1

A. Zones and areas of Costa Rica that supplied venomous snakes

B. Physical map (feet above sea level)

C. Average annual temperature (degrees Fahrenheit)

D. Annual rainfall

E. Climate (Köpen's classification)

Ecological Zones of Costa Rica

As the result of its varied elevations and location in tropical latitudes, Costa Rica, though of small area, is remarkable in its variety of climatic regions and hence number of distinct ecological zones (Fig. 1). On the eastern side of the continental divide, an Atlantic Zone, which includes the city of Puerto Limón, is a jungle region of tropical rainforest in which abundant precipitation falls in all months of the year. Three zones may be distinguished on the western side of the continental divide. A South Pacific Zone, in the region of Golfito, is tropical rainforest, but is free of precipitation from January through April. A North Pacific Zone, including the entire political province of Guanacaste, has tropical savanna climate with seasonal rainfall from May through November. A Central Pacific Zone has a similar season of rainfall but a more temperate climate. This region includes the heavily populated central plateau (Taylor, 1951; Quirós-Amador, 1954).

Costa Rican Serpent Fauna

The Costa Rican serpent fauna is large for the size of the country. Taylor (1954) lists 151 species and subspecies and arranges them in 56 genera, under 7 families. Venomous species, whose number is over a dozen, are found throughout the country, but mainly in ecological zones having tropical climates (Picado, 1931; Víquez, 1935).

The abundance of species in Costa Rica is due partly to the diverse characteristics of topography and climate, but another factor, probably of great importance, is the unique geological history of the region. Geologists (Schucher, 1935) believe that Costa Rica has been submerged at least three times since the Pennsylvanian Period of the Paleozoic. The land bridge between North and South America has thus been interrupted and re-established several times. The present land bridge between North and South America has been present for approximately one million years, since the lower pliocene, a relatively short time geologically (Fig. 2). This explains why Costa Rica is included within the range of many South and North American animals and lies directly in the path of species migrating north or south.

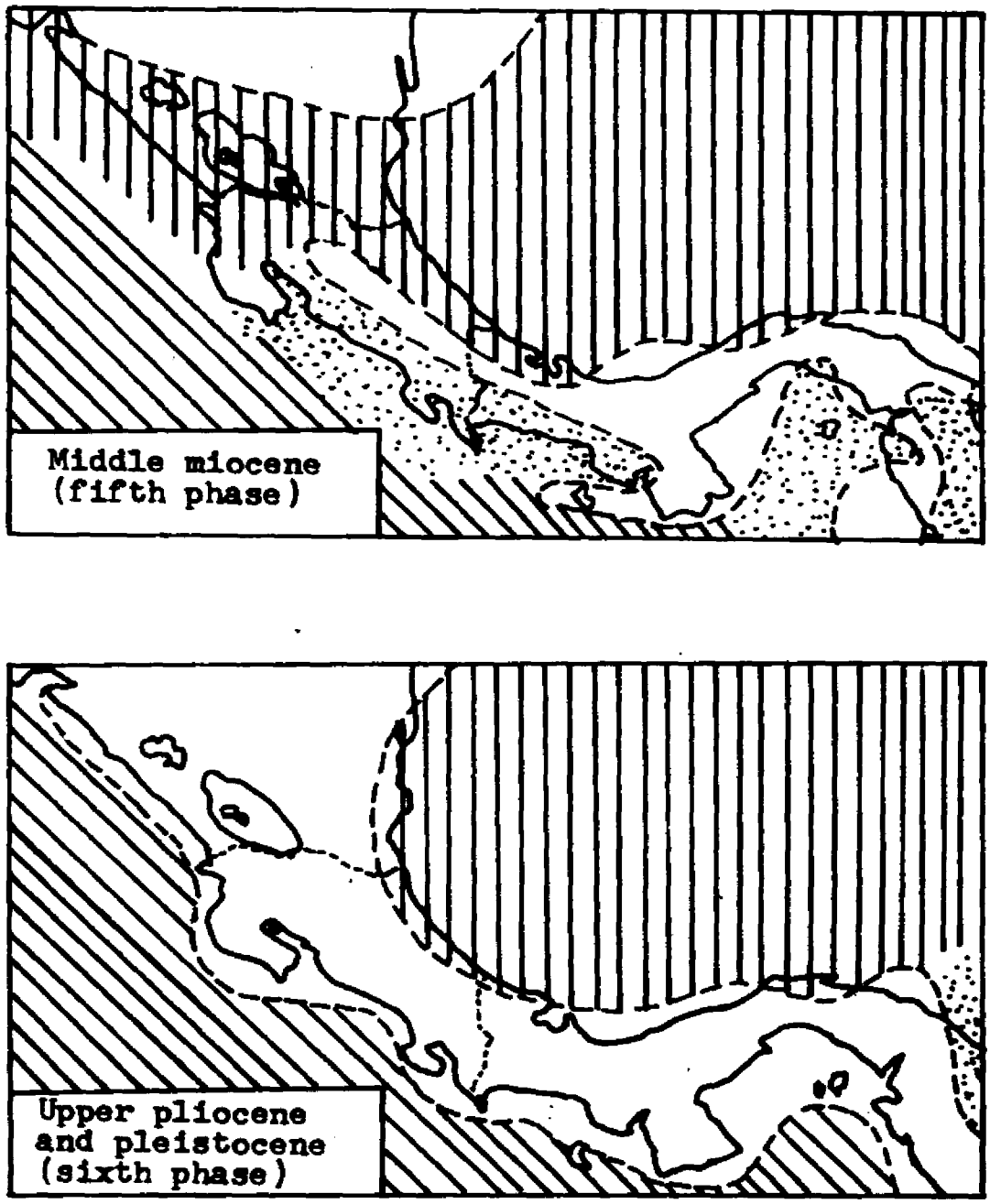


Fig. 2 Geologic evolution of Costa Rica, according to Schuchert.

Source of Animals

Snakes were obtained through the cooperation of many people. Trips were taken about Costa Rica to distribute information on venomous snakes, and to ask for help in obtaining them from country people. Leather laces to catch and handle these animals, as well as shipping containers were given to many people. Cooperation was very gratifying. Many snakes were sent to the School of Medicine by bus, train and even airplanes. To aid in obtaining animals, the following circular letter was distributed to government officers, teachers and friends throughout Costa Rica:

CIRCULAR LETTER

Ciudad Universitaria
6 de diciembre 1961

Señor

Muy estimado señor:

Estoy interesado en obtener ejemplares vivos de las siguientes serpientes venenosas de Costa Rica:

TERCIOPELO (también llamada rabo amarillo, toboba gata, toboba tiznada, etc.)

CASCABELA

CASCABELA MUDA

MANO DE PIEDRA

CORALES VENENOSAS

BOCARACA y OROPEL

Mi propósito es hacer un estudio bioquímico

comparativo de los venenos y LLEGAR A PREPARAR EN EL FUTURO SUERO CONTRA TODAS LAS ESPECIES VENENOSAS DE SERPIENTES COSTARRICENSES. Actualmente, como Ud. sabe, el llamado suero polivalente protege únicamente contra mordedura de terciopelo y cascabela.

Solicito por este medio su colaboración, que yo considero muy valiosa, para que haga saber a personas de su localidad que necesitamos serpientes venenosas vivas en esta Escuela de Medicina.

ESPECIALMENTE ESTOY INTERESADO EN OBTENER DE LA REGION DONDE UD. VIVE, EJEMPLARES DE:

.....

Me propongo visitar dentro de poco tiempo los lugares donde se localizan las serpientes en que estoy interesado y dejar unos cuantos lazos de cuero corredizos para disminuir el peligro del manejo de los animales.

Por ejemplares pequeños y relativamente abundantes pagamos de 10 a 20 colones. Por ejemplares grandes o valiosos por ser escasos o de distribución geográfica limitada, podemos pagar 25 colones o más.

En el caso especial de la Cascabela muda, que no existe en el centro del país, el precio será mayor (hasta 50 colones). Conviene insistir ante las personas que pueden encontrar esos animales, que más que el dinero que pueden recibir, tiene valor la ayuda que presten para que en el futuro no muera ni una sola persona por mordedura de serpientes venenosas de Costa Rica. En otras palabras, cualquier ayuda que se nos preste ahora, resultará en beneficio de las mismas personas que cooperen. Cuanto mejor conozcamos el veneno, más medios tendremos de tratar a las personas mordidas cuando lleguen al hospital.

Corre por mi cuenta el costo de envío del animal cuando se trate de un lugar lejano de San José, ya sea por avión u otro medio de transporte, para lo cual sugiero un cajón de madera con rendijas de tamaño prudente para la ventilación. Los envíos deben dirigirse a:

Prof. JESUS M. JIMENEZ PORRAS,

Director Cátedra de Bioquímica
Escuela de Medicina.
Universidad de Costa Rica
Cuidad Universitaria
San Pedro, Montes de Oca.

ES MUY IMPORTANTE QUE SE INDIQUE EL LUGAR EXACTO (cantón y distrito) donde se capturó el animal y que se sigan las siguientes instrucciones: Animales grandes deben introducirse dentro de un saco de manta o de guangoche, que se amarra arriba con un mamila o cáñamo y luego se pone en una caja de madera o de cartón, con huecos o rendijas para ventilación.

Animales pequeños pueden ponerse en un frasco de vidrio o en tarro de lata, que tenga agujeros en la tapa. Poner en la caja o frasco: SERPIENTE VENENOSA y el nombre y dirección arriba indicados. No es necesario ponerles nada de comer y en el saco o frasco pueden permanecer muchos días, SIEMPRE QUE SE CONSERVEN HUMEDOS, mojando el saco o echando agua en el frasco. Dejarlas en lugar de sombra. No exponerlas al sol ni a temperaturas altas ni aun durante el transporte, pues son muy sensibles a la deshidratación y calor excesivo. Además, cuando se envíe desde lejos el animal y no venga acompañado por la persona, se puede pedir al chofer o cobrador del vehículo que deposite la caja en cualquiera de las puertas del Hospital San Juan de Dios, cuando sea del todo imposible venir a la Cuidad Universitaria.

El dinero será enviado apenas se reciba el animal, SIEMPRE QUE SEA VENENOSO. (LAS SERPIENTES VENENOSAS DE COSTA RICA, CON EXCEPCIÓN DE LAS CORALES Y LAS SERPIENTES DE MAR, TIENEN UN AGUJERO TRIANGULAR ENTRE EL OJO Y EL AGUJERO NASAL, LO QUE SIRVE PARA DISTINGUIRLAS EN CASO DE DUDA). Si la persona trae el animal, se le paga al entregarlo.

En espera de que Ud. se sirva prestar la valiosa cooperación que le solicito, me suscribo su atento servidor,

Prof. Jesús M. Jáménez Porras
Director Cátedra de Bioquímica
Escuela de Medicina

METHODS

Snakes were housed in an especially designed serpentarium in the animal house of the University of Costa Rica School of Medicine. Large fer-de-lances, rattlesnakes and bushmasters were maintained in pits whose floors were covered with dirt and stone. Smaller snakes were kept in wire cages on wooden floors. Light and temperature were not controlled. A catalog number was assigned to each animal and its identity, locality of capture, weight, length, and dates of arrival, milking, feeding, etc. were recorded. Records were kept of dates of birth and identity of the mother, when possible, of snakes born in the serpentarium.

Venom was collected within 3-4 days after specimens were received. They were fed after the first milking. With the exception of some specimens of Bothrops atrox, B. schlegeli, B. godmani and Crotalus durissus, most animals refused to kill and eat white mice, hamsters and other small animals placed in their pits or cages. For this reason, such animals were force-fed. Specimens of B. godmani and newborn rattlesnakes ate mice very voraciously. Milking and feeding of each animal were usually repeated monthly.

Appendix I summarizes data on size and venom yields of the animals.

Milking Procedure

Snakes were allowed to strike through a rubber membrane stretched across the mouth of an elongated wine-glass (Jiménez-Porras, 1961). As fangs penetrated the membrane, the snake ejected its venom into the glass. In most cases, venom glands were massaged to obtain additional venom (Plate I). Venom could be collected from newborn specimens of B. nummifera and C. durissus as early as a few days after birth. Venom was analyzed in the fresh state, as quickly as possible after milking. That not used immediately was stored in a deep freeze.

Plate I. Milking Procedure

Analytical Methods

For the most part, methods were identical to those utilized in work with C. atrox venom, during my Masters research. To measure solid content, known volumes of venom (0.05-0.20 ml) were dried to constant weight over calcium chloride in vacuo. Total protein was estimated by means of the biuret method of Reinhold (1953).

Enzyme Assays

Tryptic, peptic and catheptic activities were assayed upon an urea denatured hemoglobin substrate buffered at either pH 1.6, 3.7 or 8.5 (Anson, 1939; Grassmann and Hanning, 1954). Rennin, chymotrypsin and papain were assayed by means of their action upon milk at different pHs (Sumner and Somers, 1947). L-amino acid dehydrogenase was determined by a manometric technique (Ratner, 1955); L-leucine was utilized as substrate and the gas space of the Warburg cups was filled with oxygen. Acetylcholinesterase was assayed either manometrically (Augustinsson, 1957) or colorimetrically (Gomori, 1953). All specific phosphatases were determined colorimetrically (Hurst, 1951; Taborda, et al., 1952a, b; Hepel and Hilmoe, 1955). A method based upon the hydrolysis of calcium bis (p-nitrophenyl) phosphate was utilized to determine unspecific phosphodiesterase (Sinsheimer and Koerner, 1952); hydrolysis was stopped by addition of NaOH (Yang, et al., 1958). Acid and alkaline

unspecific phosphomonoesterases were assayed by the method of Shinowara, et al. (1942). Indirect hemolysin (phosphatidase A) was assayed by the technique of Yang, et al. (1954) substituting human for rabbit erythrocytes. Results were expressed as the dilution of venom producing fifty per cent hemolysis (Stein and Van Ngu, 1950; Jiménez-Porras, 1961). Direct hemolysis was assayed by the same method, excluding lecithin from the reaction mixture. The viscosimetric method of Swyer and Emmens (1947) was used to estimate hyaluronidase; incubation of the reaction mixtures as well as determination of flow times were at 37°C; Catalase was assayed by the method of Gagnon (1959). Sodium and potassium were determined by flame photometry, using a lithium internal standard. Chloride was measured by the method of Schales and Schales (1941). Osmotic pressure was determined on 0.2 ml samples of venom, using a Fiske osmometer. pH values were obtained with a Beckman pH meter and a cup-type glass electrode.

Assay of Effects on the Clotting Process

The effects of venoms or venom fractions on the blood clotting *in vitro* were analyzed as follows;

a. To detect clot promoting or inhibitory effects, a reaction mixture containing oxalated human plasma plus calcium ions was used, according to a technique developed by Grasset and coworkers (1956).

b. When venom had an accelerating action on blood

clotting, its ability to convert fibrinogen into fibrin was tested by means of a procedure developed by Grasset and Schwartz (1955). The clottability of fibrinogen employed was always checked by adding thrombin to a solution of the protein in saline.

c. To detect ability of venom to convert prothrombin into thrombin, 0.2 ml of prothrombin solution, 0.2 ml of saline and 0.1 ml of a dilution of venom were added to a test tube and incubated at 37°C for 15 minutes. Then 0.3 ml of 0.5% solution of fibrinogen was added and the time required for a clot to form was noted. This time was compared with that of a reaction mixture containing 0.1 ml of venom, 0.4 ml of saline and 0.3 ml of fibrinogen. Requirement for calcium ions was checked by substituting 0.2 ml of 1.25% $CaCl_2$ solution for saline in the first reaction mixture. Prothrombin preparations were checked for thrombin prior to this test.

d. Fibrinolytic activity was detected by noting the time necessary for clots to liquify in the presence of dilutions of venom or venom fractions. Clots were formed from (1) mixtures of thrombin and fibrinogen (2) oxalated plasma and calcium chloride (3) plasma and venom samples or (4) fibrinogen and venom samples.

The prothrombin was isolated as needed from fresh oxalated human plasma by adsorption on barium sulfate (Surgenor and Noeriken, 1952). The activity of each preparation was checked by noting the time required for a clot

to form when 0.2 ml of the prothrombin preparation was incubated with 0.2 ml of prothrombin-free plasma, 0.1 ml of saline solution, 0.1 ml of thromboplastin and 0.2 ml of $CaCl_2$. Bovine fibrinogen and bovine thrombin used were obtained commercially.

Starch-gel Electrophoresis

Starch-gel electrophoresis was performed by methods developed by Smithies (1959a, b) with minor modifications introduced by Dessauer, et al. (1962). Special trays 44 cm long and 16.5 cm wide were used in a vertical position. The borate buffer utilized for preparing the gel as well as the bridge solution and the staining techniques were those suggested by Smithies for routine studies of serum proteins. A current of 15 mA at a potential difference of 150-160 volts during 18-24 hours was used. Electropherograms were stained for protein with amidoschwartz. Bands present on stained gels were identified by a letter, C if cathodal and A if anodal, and a number. The higher the number, the further the bond from the origin. Electrophoretic curves were obtained by means of a densitometer with a 485 mu filter at a chart velocity of one inch per minute.

To determine the enzyme activities of electrophoretic fractions, proteins were eluted from sections of the gel after electrophoresis. Stained gels, after correcting for shrinkage during staining, were used as references to

locate the position of fractions on the unstained gels. The proteins in solution in buffer were squeezed from the starch matrix after destroying the gel structure by alternate freezing and thawing. When necessary, venom fractions were concentrated and freed from buffer by means of ultrafiltration through a collodion filter. Protein content in fractions was estimated by means of a spot test, using bromphenol blue. The same techniques employed with unfractionated venoms were used to identify enzyme activities of the fractions. The bright yellow color of L-amino acid dehydrogenase also served to localize this enzyme. The position of electrophoretic fractions with trypsin activity was detected on intact gels by the yellow color formed when the gel was incubated in a synthetic trypsin substrate, benzoyl DL-arginine p-nitro-anilide (Erlanger, et al., 1961). Toxicity of fractions was determined by intraperitoneal injection of 1 ml aliquots of known protein content into adult white mice.

THE FER DE LANCE

An outstanding feature of the distribution of pit vipers in the New World is the presence of rattlesnakes in North America and their substitution by lance-head pit vipers in Central and South America. While there are no lance-head snakes in the United States, this country is the home of more than thirty species of Crotalus. Most Central and South American vipers are lance-heads (Bothrops). Approximately 3 dozen species of Bothrops are recognized (Ditmars, 1946); at least nine species are found in Costa Rica (Picado, 1931).

Bothrops atrox, the fer-de-lance, is representative of its genus in the American tropics. It is the most abundant and widely distributed snake in the New World. Fer-de-lance is a Creole-French name that refers to the lance-shaped head of the animal. Usually these snakes are slender, with a maximum length of about 2 m. The species has probably attained the highest development and efficiency in the injecting apparatus among snakes throughout the world. Length of the fangs usually exceeds one inch. The color pattern is characterized by a gray or greenish-gray

background with darker pale-edged triangles, the apex of these extending to the center of the back (Plate II). There is considerable variation in hue. Some specimens from the Atlantic Zone of Costa Rica are stout and exhibit a black pattern. (Plate III).

One hundred and sixteen individuals (Appendix I) were studied. These were obtained from many areas of all ecological zones of Costa Rica (Fig. 3).

Plate II. Most common color pattern of Bothrops atrox.

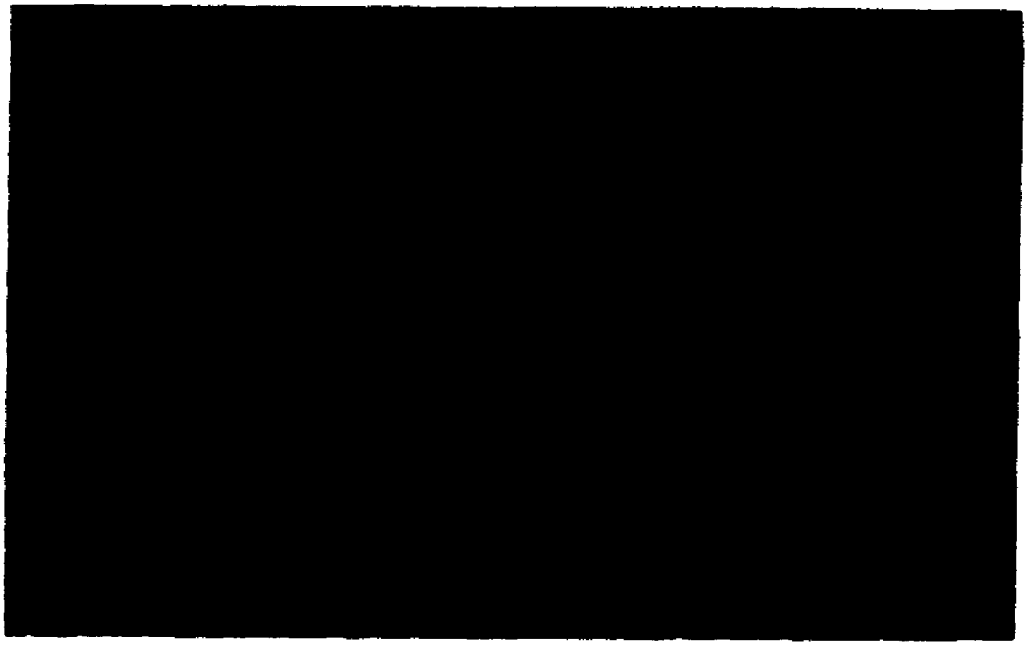


Plate III. Black variant of the fer-de-lance, found only in the Atlantic zone.

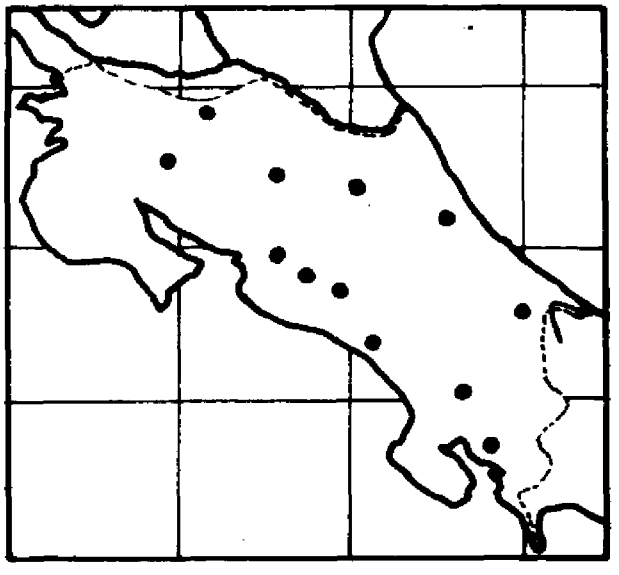


Fig. 3. Geographic distribution of specimens of Bothrops atrox studied.

Venom of the fer-de-lance

Venom was bright yellow and very viscous. This species produced larger amounts of venom than any other species studied, including the much heavier rattlesnakes and bushmasters (Appendix I). Many fer-de-lances ejected more than 2 ml per milking. One specimen from the Atlantic Zone gave 6.2 ml (1,300 mg of dried venom), a volume greater than any recorded in the literature (Porges, 1953; Minton, 1957).

Gross Composition of Venom

An average of 29% of the venom is solid. Most of the solid, an average of 83%, is protein. The percentage of the solid due to protein was relatively constant (Appendix II).

Venom has an acid reaction which varies little from its average pH of 5.8. The osmotic pressure of 339 mOsm/L is about equal that of snake blood (Dessauer, personal communication). Venom sodium (avg. 179 mEq/L) is of the same order of magnitude as plasma sodium (Dessauer, et al., 1961); potassium (17 mEq/L) is about three times higher and chloride (19 mEq/L) five times lower. Sodium and potassium varied inversely. Venoms with low sodium contained an equivalent rise in potassium and vice versa (Appendix II). Phosphate was absent.

Proteolytic Activities and Action on Blood Clotting

Proteolytic enzymes of the tryptic, chymotryptic, rennin

and papain types were found in B. atrox venom. Pepsin and cathepsin were absent (Appendices VII and IX).

Venom of B. atrox and dilutions of venom up to 100-fold prevented plasma clotting, whereas higher dilutions enhanced clotting (Appendix III). These antagonistic effects were due to simultaneous presence of thrombic and fibrinolytic substances. For example, in the presence of 100 to 1000-fold dilutions of venom, fibrinogen clotted, but the fibrin formed liquefied within 3 to 5 hours (Appendix IV). Furthermore, high concentrations of venom that prevented clotting were able to dissolve preformed clots.

At highest venom concentrations, the coagulant effect was counteracted by the fibrinolysin and the reaction mixture became incoagulable. At moderate concentrations, both effects appeared; the higher the dilution, the longer the time required to redissolve clots. At high dilutions the fibrinolytic effect disappeared and clotting time became longer, since the clot-promoting substance was also diluted.

Acceleration of clotting was also due to the presence of prothrombinase activity, which required the presence of calcium ions (Appendix V). Prothrombinase also seems to be active in vivo, since the most characteristic biochemical change observed in blood of victims of fer-de-lance is a striking decrease in prothrombin level, which returns to its normal value after administration of antiserum (Peña-Chavarría and Badilla, personal communication).

A number of other workers have studied these proteolytic and coagulant properties of fer-de-lance venom. Picado (1931) believed that the coagulant activity causes the extensive gangrene in snake bite patients. Hanut (1937, 1938) reported on the ability of B. atrox venom to convert fibrinogen directly into fibrin. Eagle (1937) also studied that property; he emphasized that the coagulant action was independent of calcium ions, platelets, or tissue extracts. He believed that it was due to the proteolytic enzymes present in the venom. Eagle also found that B. atrox venom converts prothrombin into thrombin, but according to his results, calcium ions were not necessary. He did not detect anticoagulant or fibrinolytic activities. Jánszky (1950) also reported on the coagulant properties of B. atrox venom and concluded that blood clotting was due to proteolytic enzymes.

Action on Human Erythrocytes

Neither direct hemolysins nor hemagglutinins were found in B. atrox venom (Appendix VII). On the other hand, a very potent indirect hemolysin, phosphatidase A, was present (Appendix IX). This hemolysin appeared to be as powerful as that reported for cobra venom by Yang, et al. (1954).

Other Enzymes

Hyaluronidase, L-amino acid dehydrogenase, five specific phosphatases and unspecific phosphodiesterase activities were

measured. Adenosine-5'-monophosphatase (AMP-ase), nicotinamide-dinucleotidase (NAD-ase) and adenosine-triphosphatase (ATP-ase) were the most active specific phosphatases, activity being highest for adenosine-5'-monophosphatase. Deoxyribonuclease (DNA-ase) and ribonuclease (RNA-ase) were much less active, the former being the least active specific phosphatase (Appendices VIII and IX). Acid and alkaline phosphomonoesterases, catalase and cholinesterase were absent.

Electrophoretic Fractionation

Venom from a large individual of B. atrox from San Carlos, in the Atlantic Zone, was utilized as the reference sample in electrophoretic studies. Six cathodal (C_1 - C_6) and 8 anodal (A_1 - A_8) fractions were separated from this venom. Almost all cathodal fractions and anodal fractions A_2, A_3 and A_6 were very concentrated in venom of this snake (Plate IV).

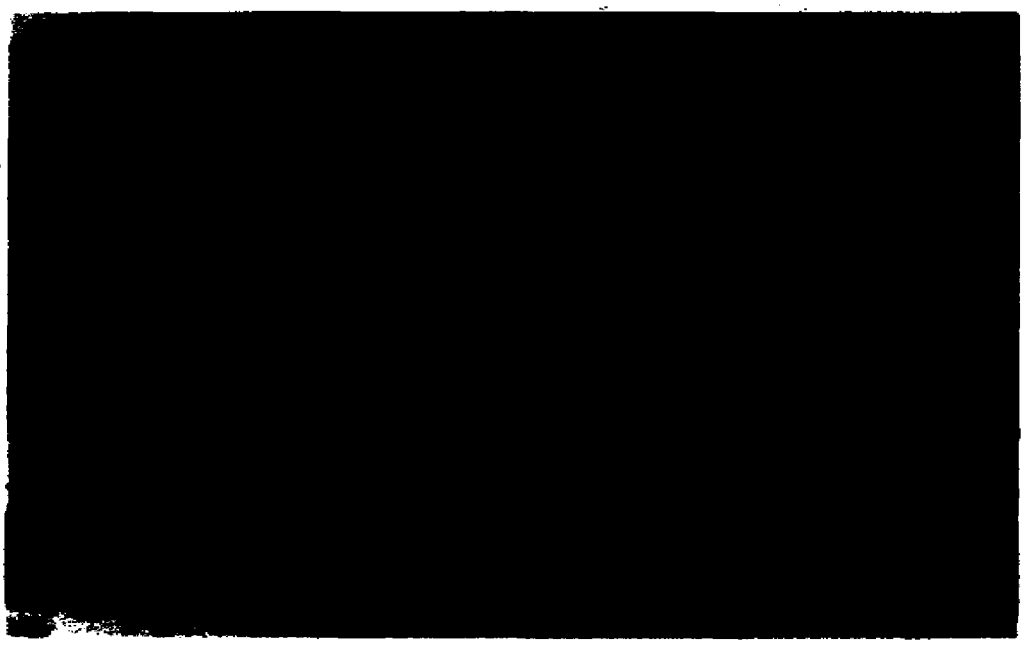


Plate IV. Starch-gel electrophoretic pattern of venom of a specimen of B. atrox from San Carlos (Atlantic Zone). Venom was diluted 2X with 0.85% NaCl prior to electrophoresis. Arrow indicates site of sample application.

Each electrophoretic fraction was assayed for all enzyme activities previously found in unfractionated venom (Table I). L-amino acid dehydrogenase was found in a yellow anodal fraction of fast migration (A_6). The two fastest anodal fractions (A_7 and A_8), containing very little protein, exhibited indirect hemolytic activity (phosphatidase). Other bands contained more than one activity. The five specific phosphatases, unspecific phosphodiesterase, papain and rennin were concentrated in two slow cathodal bands (C_1 and C_2). Chymotryptic and tryptic activities were found in the same bands that exhibited a multiple action on the clotting process. One group, combining chymotryptic with thrombic activities, moved rapidly to the cathode (C_4 and C_5); the other group, represented by 4 anodal bands (A_2, A_3, A_4 and $A_4{}^1$) of slow and intermediate speed, combined tryptic with clotting actions. Among these 4 bands, A_2 and A_3 contained the most protein and highest enzyme activities.

TABLE I

ENZYME ACTIVITIES
OF ELECTROPHORETIC FRACTIONS
OF VENOM OF A FER-DE-LANCE

Activity	C_6	C_5	C_4	C_3	C_2	C_1	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8
Phosphatases	-	-	±	±	4+	2+	±	-	-	-	-	-	-	-
L-amino acid dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Phosphatidase A	-	-	-	-	-	-	-	-	-	-	-	±	2+	4+
Trypsin	-	-	-	-	-	2+	2+	4+	3+	2+	-	-	-	-
Chymotrypsin	3+	4+	4+	2+	-	-	-	-	-	-	-	-	-	-
Papain	-	-	-	-	4+	4+	2+	-	-	-	-	-	-	-
Rennin	-	-	-	-	1+	±	-	-	-	-	-	-	-	-
Thrombin	1+	1+	4+	1+	3+	3+	3+	4+	4+	4+	2+	-	-	-
Prothrombinase	-	-	-	±	±	±	2+	2+	2+	2+	1+	±	-	-
Fibrinolysin	-	-	3+	3+	±	2+	3+	3+	2+	2+	-	-	-	-

The toxicity of fractions, adjusted to equal concentration of protein (0.3 mg/ml), was compared (Table II). Fractions C_4 and C_5, containing chymotryptic and thrombic activities were most toxic. Only one of twenty-six mice lived following injection with the fractions. The least toxic fractions were the fast anodal bands (A_6, A_7, and A_8), which included L-amino acid dehydrogenase and phosphatidases; and the slow cathodal fractions (C_1 and C_2) in which the various phosphatases were concentrated. Mortality of mice following injections with these fractions was 50 to 60 per cent.

TABLE II

TOXICITY OF ELECTROPHORETIC FRACTIONS
OF VENOM OF A FER-DE-LANCE

Fraction	No. mice injected	% mortality	Survival time (hours)	Toxicity
A_1	12	75	3-48	2+
A_2	12	67	2-8	2+
A_3	13	77	3-24	2+
A_4	12	75	3-24	2+
A_5	13	69	2-9	2+
A_6	10	60	2-8	1+
A_7	10	50	5-10	1+
A_8	10	60	3-24	1+
C_1	9	56	2-12	1+
C_2	10	50	5-24	1+
C_3	16	69	3-24	2+
C_4	15	94	3-10	3+
C_5	11	100	3-10	4+
C_6	9	56	5-24	1+

Intraspecific Variation in Venom Proteins

The fer-de-lance is particularly suitable for studies of protein variation within a species because of its great numbers and the wide extent of its range, which includes all of South and Central America. It is found in all ecological areas of Costa Rica, but is not common in the North Pacific Zone.

Geographic variation. Venom proteins of populations of fer-de-lance from numerous collecting sites in all ecological zones of Costa Rica were compared by starch gel electrophoresis. Plate V displays electropherograms run on the same gel, of venom of specimens from the Atlantic and the Central Pacific Zones. Plates VI, VII and VIII present electropherograms of additional venoms of animals in each of three ecological zones. The most common electrophoretic patterns of animals from different ecological zones are diagrammed in Figure 4.

Two major differences in patterns of animal proteins distinguished populations from the Atlantic and Pacific sides of the continental divide (Plate V). First, even on unstained gels populations of snakes from these areas could be recognized by the relative migrations of L-amino acid dehydrogenases (Table IV). This pigmented protein appeared as a single band but migrated faster in venoms of populations from the Atlantic Zone than from those of the Pacific Zones. After a twenty-four hour electrophoretic run the

Plate V. Starch-gel electrophoretic patterns of venoms from 8 specimens of B. atrox.

a, e, f, g: Atlantic Zone.
b, c, d, h: Central Pacific Zone.
x L-amino acid hydrogenase
. Tryptic activity and multiple action on blood clotting.

Venom was diluted 2X with 0.85% NaCl prior to electrophoresis. Arrow indicates site of sample application.

two dehydrogenases were separated by about 2 cm.

Second, bands with tryptic activity and with multiple effects on blood clotting, which migrated between the site of sample application and the dehydrogenases, had different patterns in populations of the various ecological zones. Four bands (A_2, A_3, A_4, A_4^1) exhibited activity in venom from the Atlantic Zone. In snakes from the Pacific zones activity was localized, commonly, in two bands of high concentration. These varied among animals of the Central and South Pacific Zones. In specimens from the Central Pacific Zone, these fractions moved at rates equivalent to bands A_4 and A_4^1; in most animals from the South Pacific Zone, activity was present in bands A_2 and A_4^1.

At least two proteins migrating faster toward the anode than L-amino acid dehydrogenase possessed phosphatidase activity. As these bands were most often of very low concentration, their migration rates and enzyme activities were difficult to analyze. However, animals from the South Pacific Zone commonly possessed three of these bands (A_7, A_8, A_9). From individual to individual (Fig. 4; Plates V, VI, VII and VIII) the number of these fast anodal fractions varied from one to three.

Forty per cent of the venom protein migrated toward the cathode. In contrast to anodal fractions, these migrating toward the cathode were uniform within the species. However, resolution of cathodal proteins was poor in the buffer used.

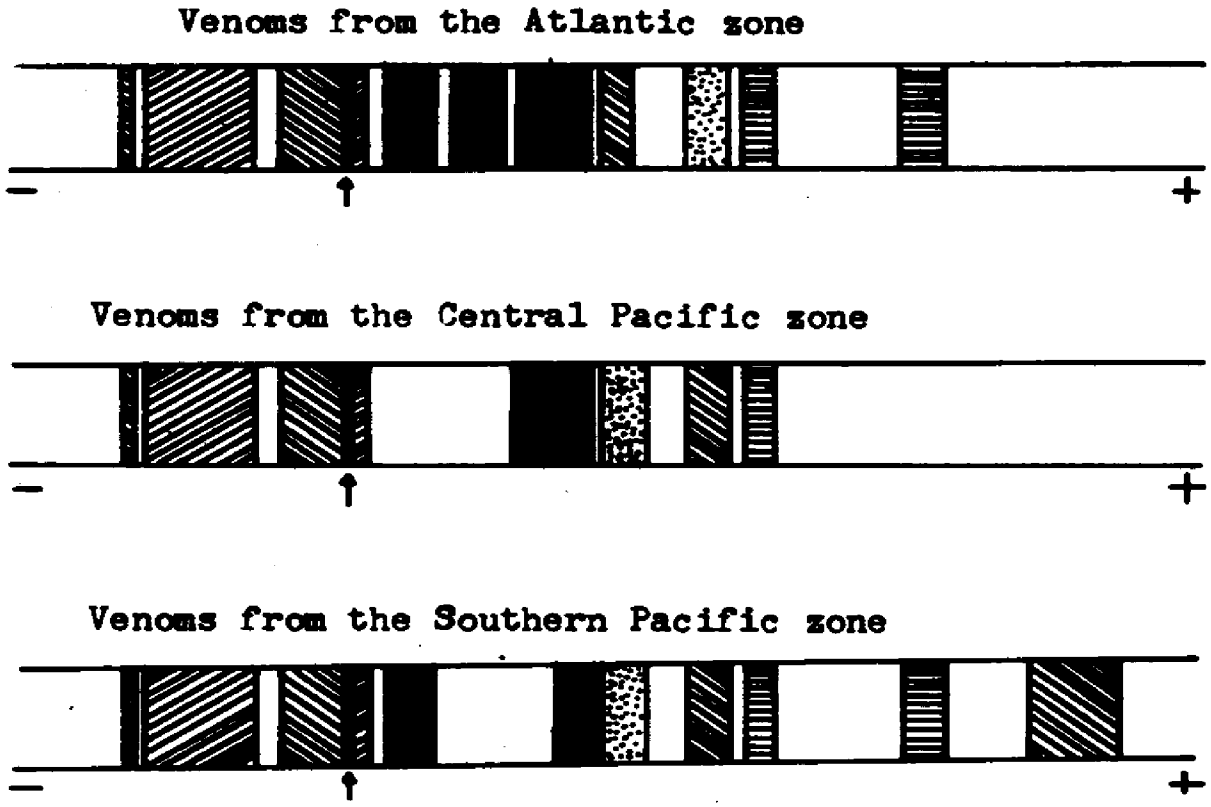


Fig. 4. A diagrammatic representation of the most common patterns of venom proteins of B. atrox from different ecological zones.

Activities of some enzymes in unfractionated venoms differed in animals from the various ecological zones. ATP-ase, DNA-ase and RNA-ase were about twice as high in specimens from the Pacific Zones than in those from the Atlantic Zone. AMP-ase and NAD-ase were lowest in venoms from the Southern Pacific Zone. Venom from the Central Pacific Zone was most hemolytic as indicated by its phosphatidase activity (Table III). Total solids, protein, osmotic pressure and inorganic composition of venoms from the three zones were not significantly different.

TABLE III

ENZYME ACTIVITIES OF VENOM FROM BOTHROPS ATROX
FROM DIFFERENT ECOLOGICAL ZONES

AMP-ase mM P/ml/h	NAD-ase mM P/ml/h	ATP-ase	DNA-ase µM P/ml/h	RNA-ase	Unspecific phosphodi-esterase
		(Atlantic Zone)			
79.4±30[a]	8.1±3.8	3.1±1.3	145±17	544±222	662±129
28.4-130[b]	4.3-18.2	1.3-6.7	120-170	352-1086	329-769
12[c]	12	12	10	12	12
		(Central Pacific Zone)			
69.1±28	11.0±5.5	7.8±1.2	422±89	1195±437	400±70
31.0-137	4.7-22.5	5.2-9.4	332-650	682-2120	312-594
19	19	19	19	17	18
		(South Pacific Zone)			
47.4±23.6	6.2±3.7	6.0±1.4	380±73	1115±426	570±10.3
24.0-110	4.0-15.9	4.2-8.8	310-533	634-1820	446-710
11	11	11	11	10	10

[a] Avg. ± S.D. [b] Range [c] No. of samples

TABLE III (Contd.)

ENZYME ACTIVITIES OF VENOM FROM BOTHROPS ATROX

FROM DIFFERENT ECOLOGICAL ZONES

Trypsin[a]	L-amino[b] acid dehydrogenase	Phosphatidase A HD_{50}
	(Atlantic Zone)	
225± 85[c]	32.0±4.1	1 : 257,000
140-398[d]	27.2-39.8	1:266,000-1:592,000
12[e]	11	10
	(Central Pacific Zone)	
262± 62	27.5±5.2	1 : 715,000
193-400	17.0-41.6	1:320,000-1:2,664,000
18	20	21
	(South Pacific Zone)	
274± 52	30.1±7.4	1 : 377,000
185-384	22.6-43.4	1:121,000-1:500,000
10	11	10

[a] Trypsin activity is expressed in µM tyrosine released per milliliter of venom in 10 min.

[b] L-amino acid dehydrogenase in mM O_2/ml/hr.

[c] Avg. ± S.D. [d] Range [e] No. of samples

Individual Variation. Individual fer-de-lances, members of a population from a limited area, could often be distinguished from each other by differences in electrophoretic characteristics of their venom proteins. For example, venom from 16 animals captured within a 30 mile radius of Sarapiqui in the Atlantic Zone exhibited 7 different electrophoretic patterns (Plates V and VI). The typical Atlantic Zone pattern previously described (Fig. 4) characterized 7 specimens (f and g of Plate V; a, b, c, and f of Plate VI). Patterns of seven other electropherograms were slight modifications on the typical pattern. In three of these, band A_5 resolved into two bands (a of Plate V); in two others, band A_4^1 was very concentrated, and two extra fast bands of unknown activity migrated toward the anode (g of Plate VI). One venom lacked band A_2 (e of Plate V); another lacked band A_4, but an additional fraction separated between bands A_4^1 and A_5 (d of Plate VI). Two animals exhibited the slow L-amino acid dehydrogenase. One of these electropherograms was otherwise identical to the typical Atlantic Zone pattern (e of Plate VI). The pattern of the other animal was indistinguishable from that of specimens captured in the Central Pacific Zone (h of Plate VI).

Plate VI. Starch-gel electrophoretic patterns of venoms from 8 specimens of B. atrox from the Atlantic Zone.

x L-amino acid dehydrogenase
. Tryptic activity and multiple action on blood clotting.

Venom was diluted 2X with 0.85% NaCl prior to electrophoresis. Arrow indicates site of sample application.

Among 25 animals captured within a 15-mile radius of Puriscal in the Central Pacific zone, the most common electrophoretic pattern (Fig. 4) was characterized by 8 specimens (g, h of Plate V). Four venoms showed extra anodal bands, fairly intense and of fast migration (c of Plate VII). In one venom there was a band at an intermediate position between A_2 and A_3 (d of Plate VII). In 4 specimens band A_4 was very slight (d of Plate V; a, g, h of Plate VII). In one specimen band A_4 was absent (f of Plate VII). Three venoms showed band A_2, although slight, as in the most common pattern in snakes of the South Pacific Zone (b of Plate V; b of Plate VII). Four animals exhibited electrophoretic patterns with 2 yellow bands (L-amino acid dehydrogenases), the fast and slow types.

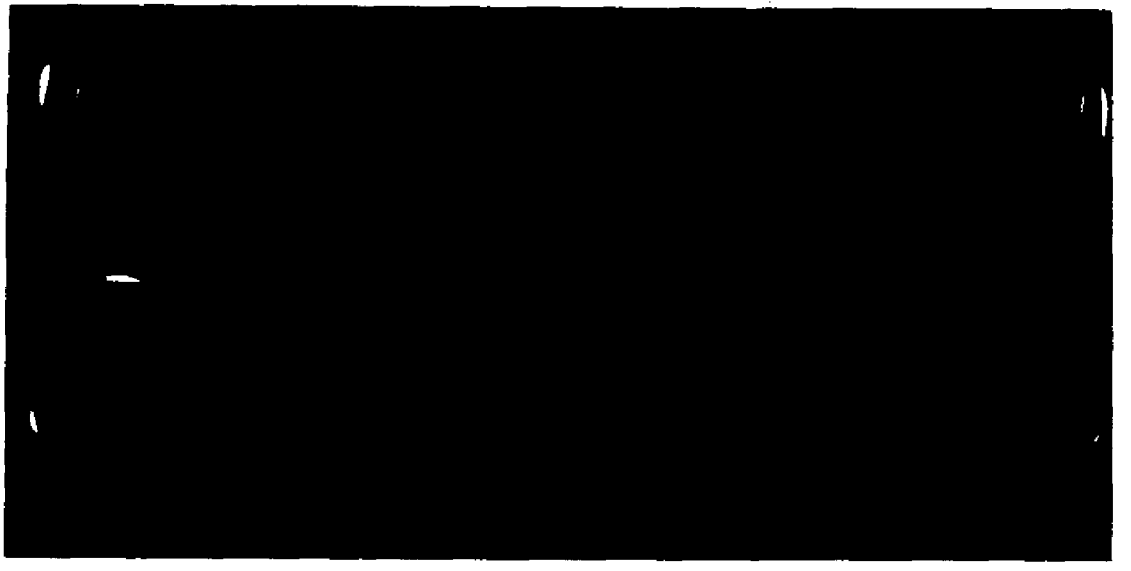


Plate VII. Starch-gel electrophoretic patterns of venoms of 8 specimens of B. atrox from the Central Pacific Zone.

x L-amino acid dehydrogenase
• Tryptic activity

Venom was diluted 2X with 0.85% NaCl prior to electrophoresis. Arrow indicates site of sample application.

Venom from 9 animals captured within the South Pacific Zone exhibited 5 different electrophoretic patterns (Plate VIII). The most common pattern of the South Pacific Zone previously described (Fig. 4) characterized 3 animals (d, f of Plate VIII). Three other venoms lacked the extra fast anodal bands (b, e, g of Plate VIII). One individual showed an extra cathodal band, very fast and not found in any other fer-de-lance (h of Plate VIII). One venom showed absence of band A_2 and C_2 (a of Plate VIII). One venom exhibited the pattern most common in the Atlantic zone (c of Plate VIII).

Electrophoretic patterns of venom proteins, though variable between animals of the different ecological zones, indicate that fer-de-lances of Costa Rica are of one species. Individuals from the different ecological zones probably intergrade to some extent. For example, although the fast type of the L-amino acid dehydrogenase is most common in the Atlantic Zone and the slow type in the Pacific Zones, both dehydrogenases are present in populations of all ecological zones (Table IV). The four individuals of the Central Pacific Zone, whose venom contains both fast and slow dehydrogenases, probably inherited factors controlling the synthesis of both proteins from their parents. The color variants (Plate III), which are found only in the Atlantic Zone, all exhibited the common pattern of animals of that region (Fig. 4). However, many of the typically colored snakes also produced venom having this pattern.

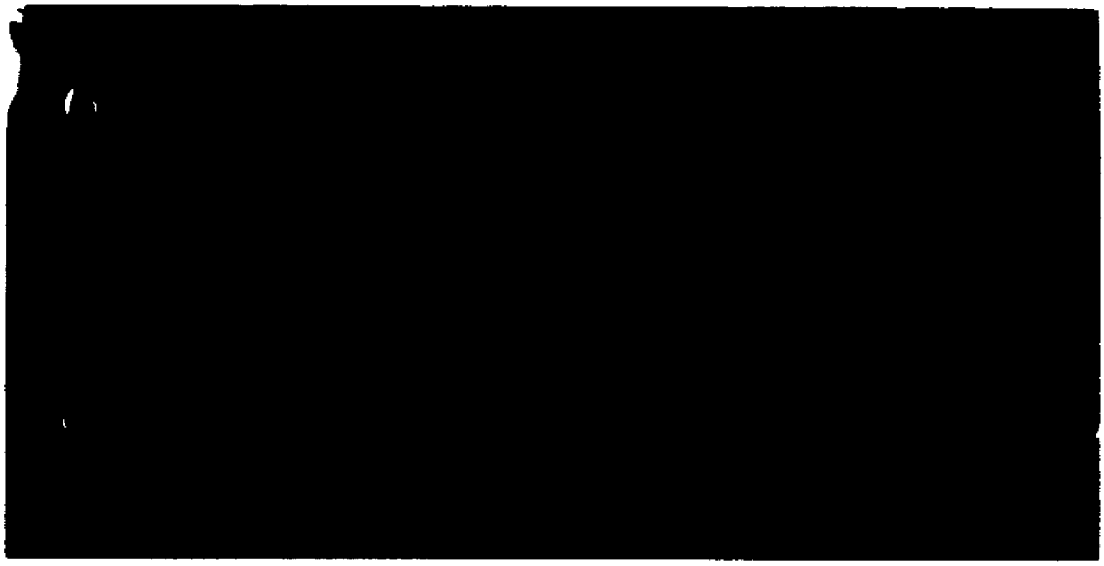


Plate VIII. Starch-gel electrophoretic patterns of venoms from 8 specimens of B. atrox. Diluted two-fold with 0.85% NaCl solution.
South Pacific zone (area of Golfito)

x L-amino acid dehydrogenase.

• Tryptic activity

TABLE IV

DISTRIBUTION OF L-AMINO ACID DEHYDROGENASE

Ecological Zone	No. Animals	Phenotype			Gene frequencies[a]	
		Slow	Fast	Fast & Slow	Slow	Fast
Atlantic	17	2	15	0	13%	87%
Central Pacific	25	21	0	4	92%	8%
South Pacific	9	8	1	0	89%	11%

[a]assuming that each protein is determined by one of a pair of genes.

Since so many variations were observed among the different fer-de-lance venoms, questions could be raised as to whether the variations were artificial. A number of observations indicate that these are not artifacts. One specimen of fer-de-lance was sampled many times over a period of three months. Samples were frozen from one to three months and then compared to freshly obtained venom (Plate IX). No major differences were found between the electrophoretic patterns of these samples. Further, the eight animals whose electrophoretic patterns appear in Plate VI were milked again after three months and their patterns again compared. No changes were observed in the number of bands or their relative migration. The individual from the South Atlantic Zone whose venom contained a unique fast cathodal band was sampled numerous times over a four month period. The unique band as well as the other bands of the electrophoretic pattern were identical in all samples.

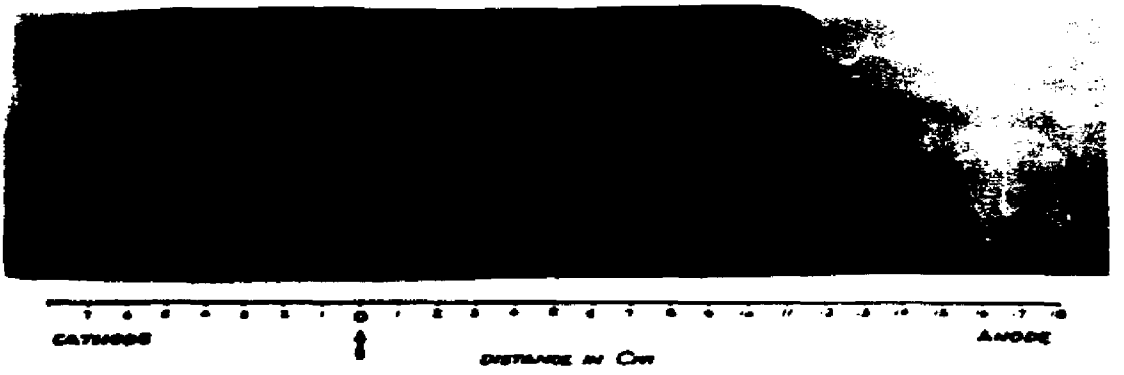


Plate IX. Electrophoretic patterns of 6 samples of venom produced by one big specimen of B. atrox from the Atlantic Zone. Samples were kept in the freezer for the following periods (days):
a- 83 b- 72 c- 60 d- 33 e- 27
f- freshly collected.

THE JUMPING VIPERS

Jumping vipers, in contrast to fer-de-lances, are short snakes that seldom reach more than three feet but have the stoutest bodies and largest heads of any species of Bothrops. Their scales are exceedingly rough. Their color pattern is commonly gray or brown with dark rhomboid blotches. The common name of these savage snakes originates from their habit of making a short jump toward their victims. Two species, both of which occur in Costa Rica are recognized. These are found most commonly in the cool and humid habitat of moderate elevations.

Bothrops nummifera

Bothrops nummifera is the most common jumping viper. Its range includes Southern Mexico and all of Central America. The species occurs throughout Costa Rica but is least plentiful in the North Pacific Zone. Color of the snakes varies on the Atlantic and Pacific sides of the continental divide. Specimens from the Atlantic Zone are gray; those from Pacific Zones are reddish (Plate X). Twelve adults and six juveniles, obtained from seven areas of the ecological

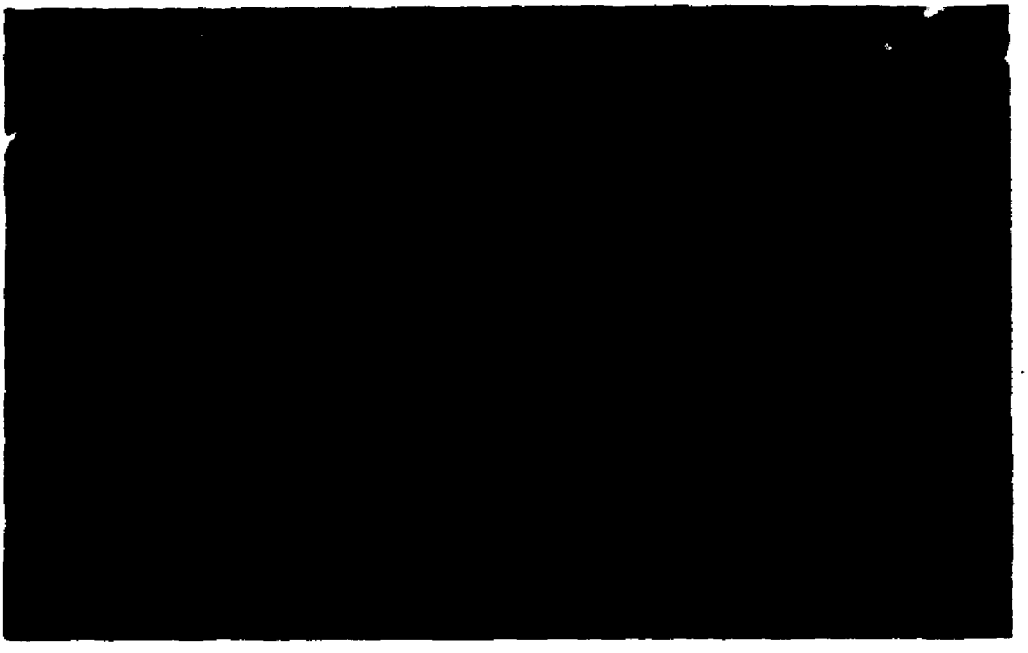


Plate X. Bothrops nummifera

Above: from the Atlantic Zone

Below: from the Pacific Zone

zones of Costa Rica were studied (Fig. 6). Venoms from twenty-seven individuals, born in captivity, were also studied.

Venom Composition

Venom of this species varied from colorless to bright yellow and was less viscous than venom of the fer-de-lance. Quantity of venom obtained in individual milkings was less than from B. atrox (Appendix I). About one-fifth of this venom was solid, of which proteins represented about 70 per cent; pH was in the neighborhood of 5.9. Potassium, averaging 150 mEq/L, was very high, about three times the concentration of sodium. Commonly the reverse ratio is true in the Crotalidae. In only 6 of 25 venom samples was potassium below 150 mE/L. One of these samples contained above 95 mEq of sodium (Fig. 5). Chloride was low and phosphate absent (Appendix II).

Effects on Blood Clotting and Action on Human Erythrocytes

Both promoting and inhibitory actions on blood clotting were detected in this venom. These effects proved to be of thrombic and fibrinolytic characters, respectively. No prothrombinase activity was found (Appendices III, IV and V). Eagle (1937) previously reported the clot promoting activity.

B. nummifera venom possessed mild direct and indirect hemolytic activities and agglutinated human erythrocytes.

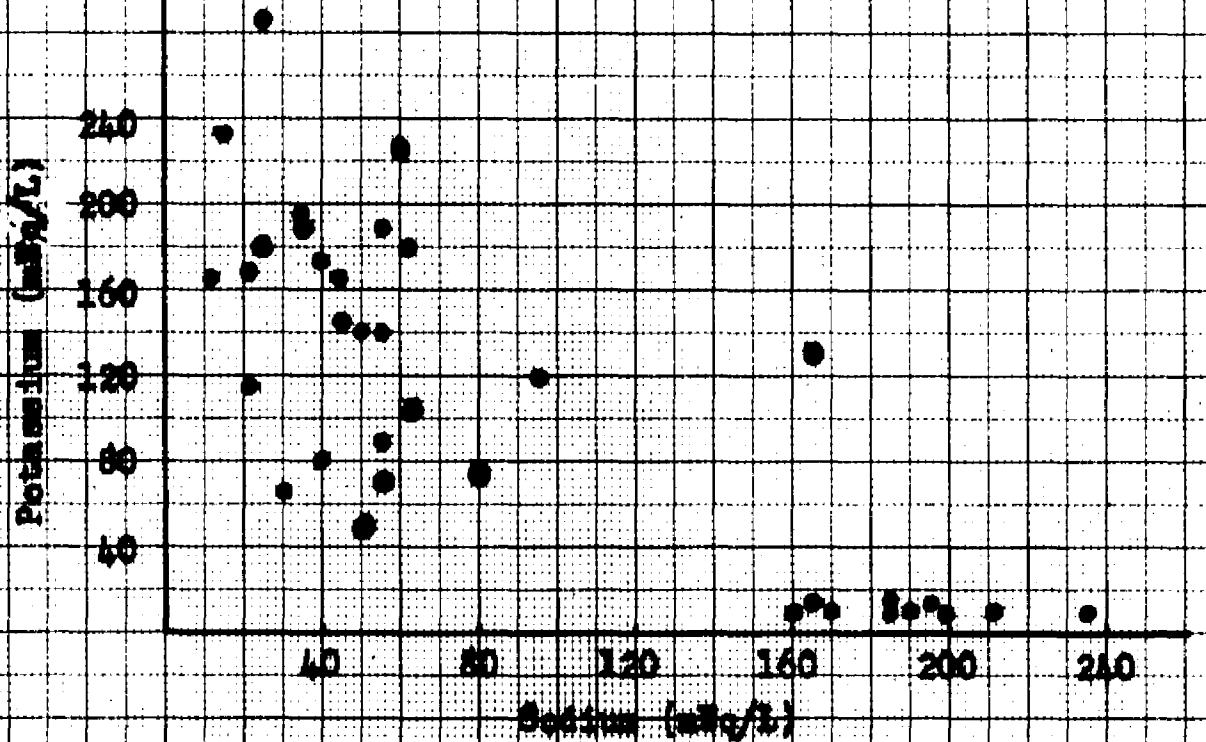


Fig. 5. Sodium and Potassium levels of venoms of individual jumping vipers.

- ● B. [redacted] (Pacific Zone)
- ● B. [redacted] (Atlantic Zone)
- ○ B. [redacted]

This venom has been reported to agglutinate and hemolyze rabbit red blood cells (Picado, 1931), but there are no reports about its action on human erythrocytes. Red blood cell agglutination was so intense that cells appeared as a solid mass after incubation in a solution containing venom. Direct hemolytic activity almost disappeared when the venom was diluted about 100-fold but phosphatidase remained active up to a dilution as high as 10,000-fold.

Other Enzyme Activities

B. nummifera venom possessed the other enzyme activities found in B. atrox venom. Adenosine-5^1-monophosphatase was the most active specific phosphatase. The other phosphatases were very feeble, particularly deoxyribonuclease and ribonuclease. In seven of 17 samples analyzed no deoxyribonuclease was found; three of 19 samples contained no ribonuclease (Appendices VIII and IX). Pepsin, cathepsin, acid and alkaline phosphatases, catalase and cholinesterase were absent.

Electrophoretic Fractionation

The earliest electrophoretic comparisons of venom proteins of B. nummifera showed that the patterns of specimens from the Atlantic Zone and Pacific Zones were markedly different. About 70% of the total protein of venoms from the Atlantic Zone migrated toward the anode whereas only 30%

was anodal in venoms from the Pacific Zones (Plate XI). Therefore, venoms from one large individual from the Atlantic Zone and another large individual from the South Pacific Zone were utilized as references in electrophoretic studies.

Geographic Variation

The striking difference between venom electrophoretic patterns of B. nummifera specimens from the Atlantic and Pacific Zones involved the most highly concentrated fraction of the patterns. This fraction migrated in a slow anodal band (A_1) in venoms from the Atlantic Zone, but it migrated in one fast cathodal band (C_3) in venoms from the Central Pacific Zone and in two fast cathodal bands in venom from the South Pacific Zone (Plates XI and XII). The position occupied by the intense band on patterns of animals from one zone contained very little protein on patterns of animals from the other zone. One of my most exciting experiences during the course of these studies was to find that these proteins, of widely different mobilities, were the most toxic fractions of the venom and contained all of its proteolytic and fibrinolytic activities (Tables V, VI and VII).

The rest of the electrophoretic fractions (Plate XI; Tables V and VI), including a distinct yellow band, L-amino acid dehydrogenase (A_3), of venoms from the two regions were identical. Phosphatidase activity, the fastest anodal band

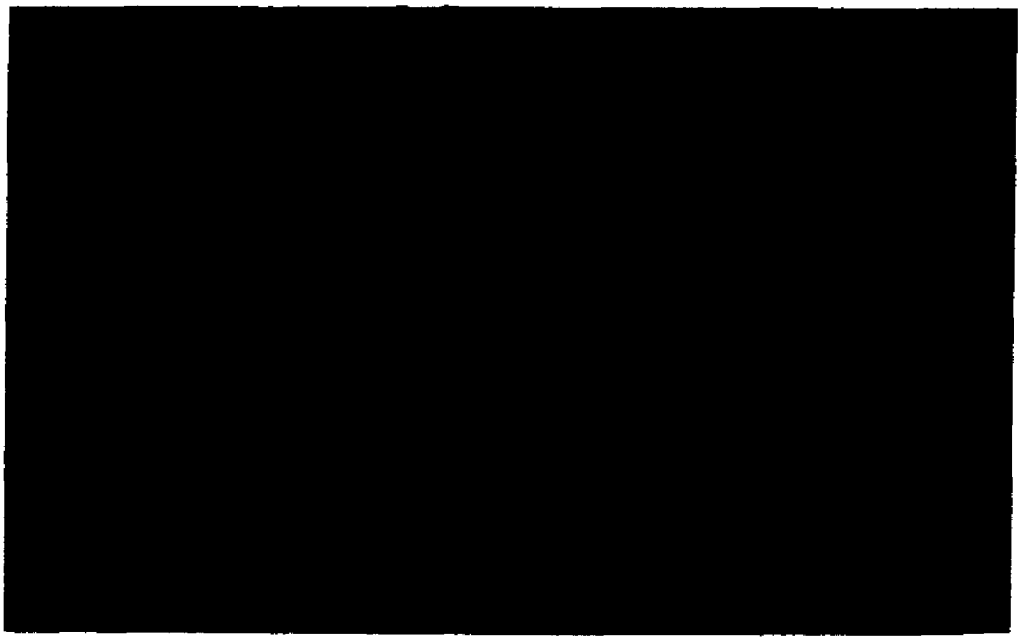


Plate XI. Starch-gel electrophoretic pattern of B. nummifera venom.

Above: Atlantic Zone.
Below: South Pacific Zone.

TABLE V

ENZYME ACTIVITIES OF ELECTROPHORETIC FRACTIONS OF VENOM OF BOTHROPS NUMMIFERA (ATLANTIC ZONE)

Activity	Electrophoretic Fraction									
	C_4	C_3	C_2	C_1	A_1	A_2	A_3	A_4	A_5	A_6
Phosphatases	-	±	3+	4+	-	-	-	-	-	-
L-amino acid dehydrogenase	-	-	-	-	-	-	+	-	-	-
Phosphatidase A	-	-	-	-	-	-	-	-	-	+
Trypsin	2+	±	2+	3+	4+	±	-	-	-	-
Chymotrypsin	1+	-	-	-	1+	-	-	-	-	-
Papain	3+	1+	2+	4+	4+	-	-	-	-	-
Rennin	4+	-	1+	4+	4+	3+	±	-	-	-
Thrombin	-	-	-	±	-	±	±	4+	±	±
Fibrinolysin	-	-	2+	4+	2+	-	-	-	-	-
Toxicity	2+	2+	2+	3+	4+	±	±	±	±	1+

TABLE VI

ENZYME ACTIVITIES OF ELECTROPHORETIC FRACTIONS
OF VENOM OF BOTHROPS NUMMIFERA (SOUTH PACIFIC ZONE)

Activity	Electrophoretic Fraction									
	C_4	C_3	C_2	C_1	A_1	A_2	A_3	A_4	A_5	A_6
Phosphatases	±	±	4+	3+	±	-	-	-	-	-
L-amino acid dehydrogenase	-	-	-	-	-	-	+	-	-	-
Phosphatidase A	-	-	-	-	-	-	-	-	-	+
Trypsin	3+	4+	±	±	±	-	-	-	-	-
Chymotrypsin	+	-	-	-	-	-	-	-	-	-
Papain	+	±	-	-	-	-	-	-	-	-
Rennin	+	+	-	-	-	-	-	-	-	-
Thrombin	-	-	-	±	-	±	±	4+	±	±
Fibrinolysin	4+	4+	±	±	±	-	-	-	-	-
Toxicity	3+	4+	1+	-	-	±	±	±	±	1+

TABLE VII

TOXICITY OF ELECTROPHORETIC FRACTIONS OF *BOTHROPS NUMMIFERA* VENOM

Fraction	No. mice injected	% mortality	Survival time (hours)	Pathology	Toxicity
			ATLANTIC ZONE		
C_4	12	42	3-4		2+
C_3	19	42	3-4		2+
C_2	21	48	1-4		2+
C_1	22	50	1-3	Lung hemorrhage	3+
A_1	31	97	1/2 - 3	Lung hemorrhage	4+
A_2	20	10	6-24		±
A_3	18	4.4	6-24		±
A_4	21	4.8	6-24		±
A_5	24	8.5	6-24		±
A_6	21	33	4-24		1+
			PACIFIC ZONE		
C_4	9	78	1/2 - 3	Lung hemorrhage	3+
C_3	7	100	1/2 - 3	Lung hemorrhage	4+
C_2	3	67	48		1+
C_1	3	0			-
A_1	3	0			-

(A_6), was a weak fraction. The five specific phosphatases and phosphodiesterase were concentrated in two slow cathodal bands (C_1 and C_2). An anodal fraction (A_4), neither toxic nor proteolytic, exhibited thrombic activity. No significant differences in intensities of enzyme activities, of clotting effects, hemolysins or agglutinins were found in unfractionated venom of animals from the various ecological zones.

Individual Variation

Very little variation in venom proteins was observed between individuals of the same ecological zone in this species. Bands C_2, C_3 and C_4, usually very weak in venoms of the Atlantic Zone, were absent in some samples (e through h, Plate XII). Venom from one specimen from the South Pacific Zone exhibited one extra cathodal band that migrated faster than C_4.

Physiological and Developmental Variations

During their time in captivity, venom samples obtained from 6 adult and 6 juvenile specimens of Bothrops nummifera were colorless or only slightly yellow and contained relatively low solid and protein. Such venoms invariably showed a much lower L-amino acid dehydrogenase activity (Tables VIII and IX) and a much weaker yellow band (A_3) on electrophoresis than yellow venoms (Plate XIII). No increase in activity resulted when flavin adenine dinucleotide was added to the

Plate XII. Geographic and individual variations in electrophoretic patterns of B. nummifera venom.

a, b: South Pacific Zone
c, d: Central Pacific Zone
e through h: Atlantic Zone
x L-amino acid dehydrogenase
• Proteolytic activities
o Phosphatases

TABLE VIII

COMPARISON OF COLORLESS AND YELLOW VENOMS PRODUCED BY B. NUMMIFERA (NEWBORN, JUVENILE AND ADULT)

	No animals	Colorless or slightly yellow	Bright yellow
Solid content (g/100 ml)	6	10.1±2.0[a] (7.2-13.3)[b]	19.9±3.8 (15.4-27.8)
Protein content (g/100 ml)	8	4.6±3.0 (2.4-11.1)	14.0±3.4 (10.3-20.9)
Osmotic pressure (m.Osm/L)	10	118±100 (6-335)	322±12 (250-396)
Trypsin (µM tyr./ml/10 10 min.)	13	230±83 (95-350)	270±33 (231-370)
L-amino acid dehydrogenase (mMO_2/ml/hr)	12	5.5±2.7 (0.1-8.5)	25.7±4.2 (18.9-31.4)
Phosphatidase A (HD_{50})	13	1:1,300 (1:606-1:6,400)	1:5,420 (1:4448-1:19040)
AMP-ase (mMP/ml/hr)	16	24.7±25.5 (0-75.6)	27.3±22.3 (8.6-112)
NAD-ase (mMP/ml/hr)	13	0.7±0.4 (0.1-1.4)	1.3±0.7 (0.2-3.3)
ATP-ase (mMP/ml/hr)	12	0.3±0.3 (0.1-0.9)	0.5±0.3 (0.1-1.2)
RNA-ase (µMP/ml/hr)	15	28.3±29.2 (0-74.1)	101.5±87 (0-326)
DNA-ase (µMP/ml/hr)	14	3.5±4.0 (0-11.3)	32.2±30.4 (0-103.6)
Unspecific phosphodiesterase (µM p-nitrophenol/ml/5 min.)	14	10.3±5.4 (2.5-22.8)	17.0±11.8 (5.8-60.0)

[a] Avg.±S.D. [b] Range

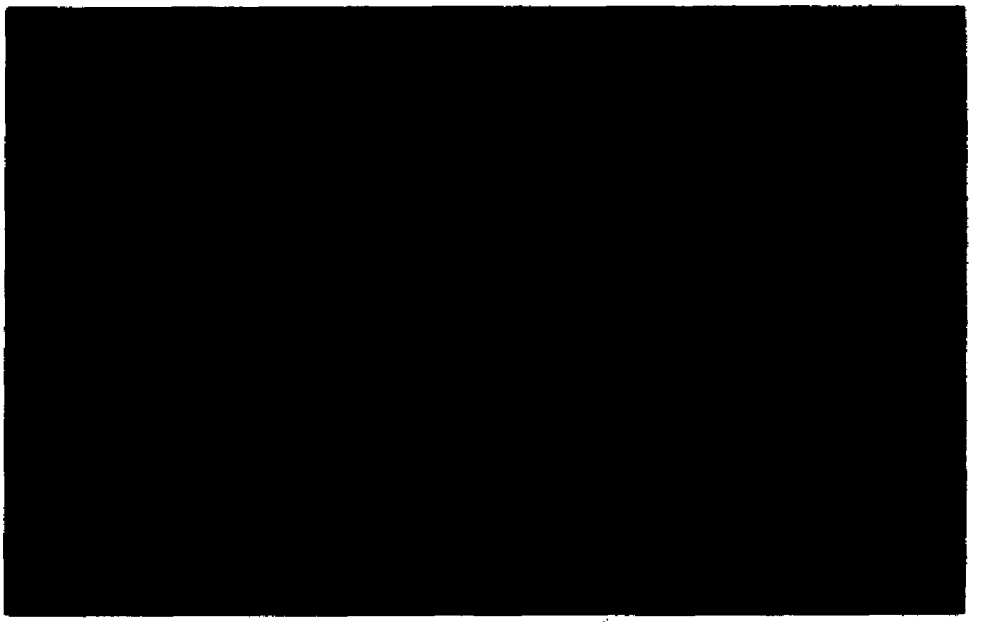


Plate XIII. Comparison of electrophoretic patterns of yellow and colorless venoms from B. nummifera.

a. Yellow sample; adult specimen from the Pacific Zone.
b. Yellow sample; adult specimen from the Atlantic Zone.
c. Colorless sample from specimen that 4 months later produced pattern a.
d. Colorless sample from specimen that 3 months later produced pattern b.
e. Slightly yellow sample from another adult specimen from the Atlantic Zone.
f. Completely colorless venom from 2 juvenile specimens from the Atlantic Zone.

assay mixture (Table IX). Similarly, other enzymes, with the exception of tryptic activity, were less active in the colorless venoms and the electrophoretic fractions in which they migrated were weaker or even absent. The diminution of activities of phosphatases was more pronounced for deoxyribonuclease and ribonuclease. Tryptic activity was almost as high as in yellow venoms, as predicted from the electrophoretic patterns (Table VIII). Out of a total of 13 samples analyzed, only one exhibited a value of tryptic activity below 100 μM of tyrosine released and only five samples showed values below 200 μM. These lower activities were correlated to electrophoretic behavior, which showed that bands A_1 of these particular samples were weaker than in colorless venoms possessing normal tryptic activity.

Twenty-seven specimens of B. nummifera were born of one mother in captivity. Fifteen of these produced colorless and twelve produced slightly yellow venom when milked one week after birth. The color of venom obtained from these newborns remained unchanged after two months in captivity; however, they had not grown. Enzyme activities of these samples were averaged in Table VIII with those of adults producing colorless venom, as no significant differences in activities were found between the two age groups. One apparent difference between the electrophoretic patterns of colorless venoms of adult and newborn was noted. The yellow band A_3, the fraction with L-amino acid dehydrogenase activity in colored venoms, was of moderate concentration

TABLE IX

EFFECT OF ADDITION OF FLAVIN ADENINE DINUCLEOTIDE ON L-AMINO ACID DEHYDROGENASE ACTIVITY OF COLORLESS AND SLIGHTLY YELLOW VENOMS OF *B. NUMMIFERA*

Specimen	Enzyme rate (mM O_2/ml/hr.)	
	Before FAD	After FAD[a]
newborn	4.8	4.1
newborn	7.7	5.9
newborn	2.8	2.5
newborn	2.8	2.7
newborn	0.1	1.9
newborn	4.5	0.5
newborn	8.2	
adult	7.2	4.4
adult	7.3	5.7
adult	4.1	3.6
adult	8.4	
adult	8.5	

[a] 20 µg added per ml of reaction mixture.

in newborn but uncolored (Plate XIV). Perhaps the apoenzyme is produced by the young; however, direct addition of flavin adenine dinucleotide to the assay mixture did not significantly increase activity (Table IX).

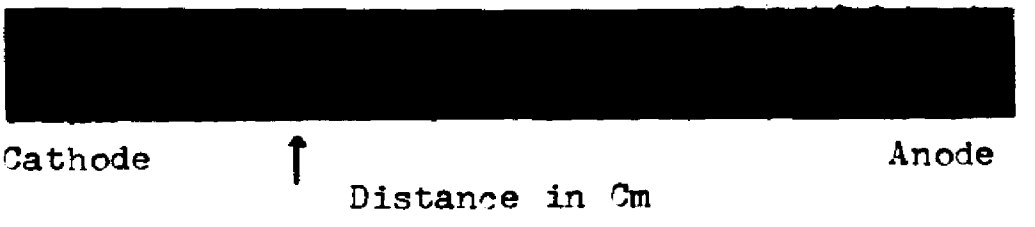


Plate XIV. Electrophoretic patterns of venom from newborn specimens of B. nummifera.

Above: slightly yellow venom
Below: colorless venom

Bothrops picadoi

Bothrops picadoi is so closely related to B. nummifera in shape, color pattern and other morphological characteristics that it is difficult to distinguish one species from the other (Plate XV). Herpetologists (Vial, personal communication) consider the safest morphological criterion distinguishing these two species to be the presence of a row of small intercalated scales on both sides of the head of B. nummifera that separates its rostral and nasal scales. Such are absent from B. picadoi (Taylor, 1951) (Plate XVI). This species appears to be restricted in its range to high elevations in the Atlantic Zone (Fig. 6). A population of 4 animals from Sarapiquí was studied (Appendix I).

Venom Composition

Venom from the four animals studied was bright yellow and viscous. One-fifth to one-fourth of this venom was solid, of which proteins represented 71% to 79%. pH was around 5.7. Unlike B. nummifera, but like other Crotalidae, sodium was high and potassium low. Chloride concentration was low and phosphate was absent (Appendix II).

Enzyme Activities

B. picadoi venom possessed mild direct and indirect hemolysins and human red blood cell agglutinins. It lacked acid and alkaline phosphomonesterases, catalase and cholinesterase. Adenosine-5'-monophosphatase was the most

Plate XV. Bothrops picadoi

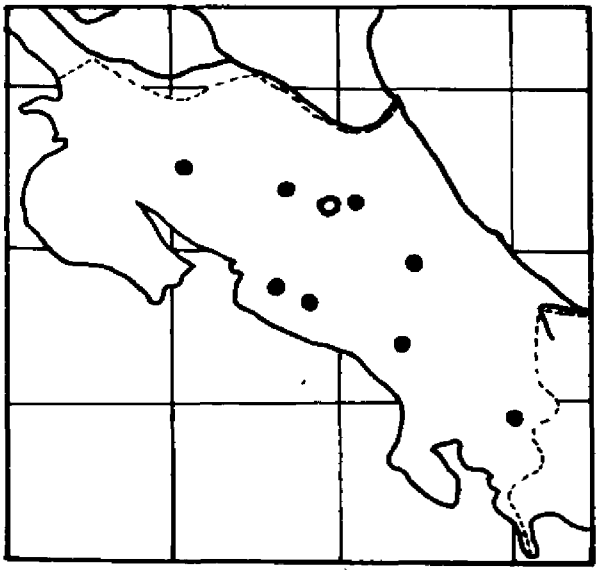
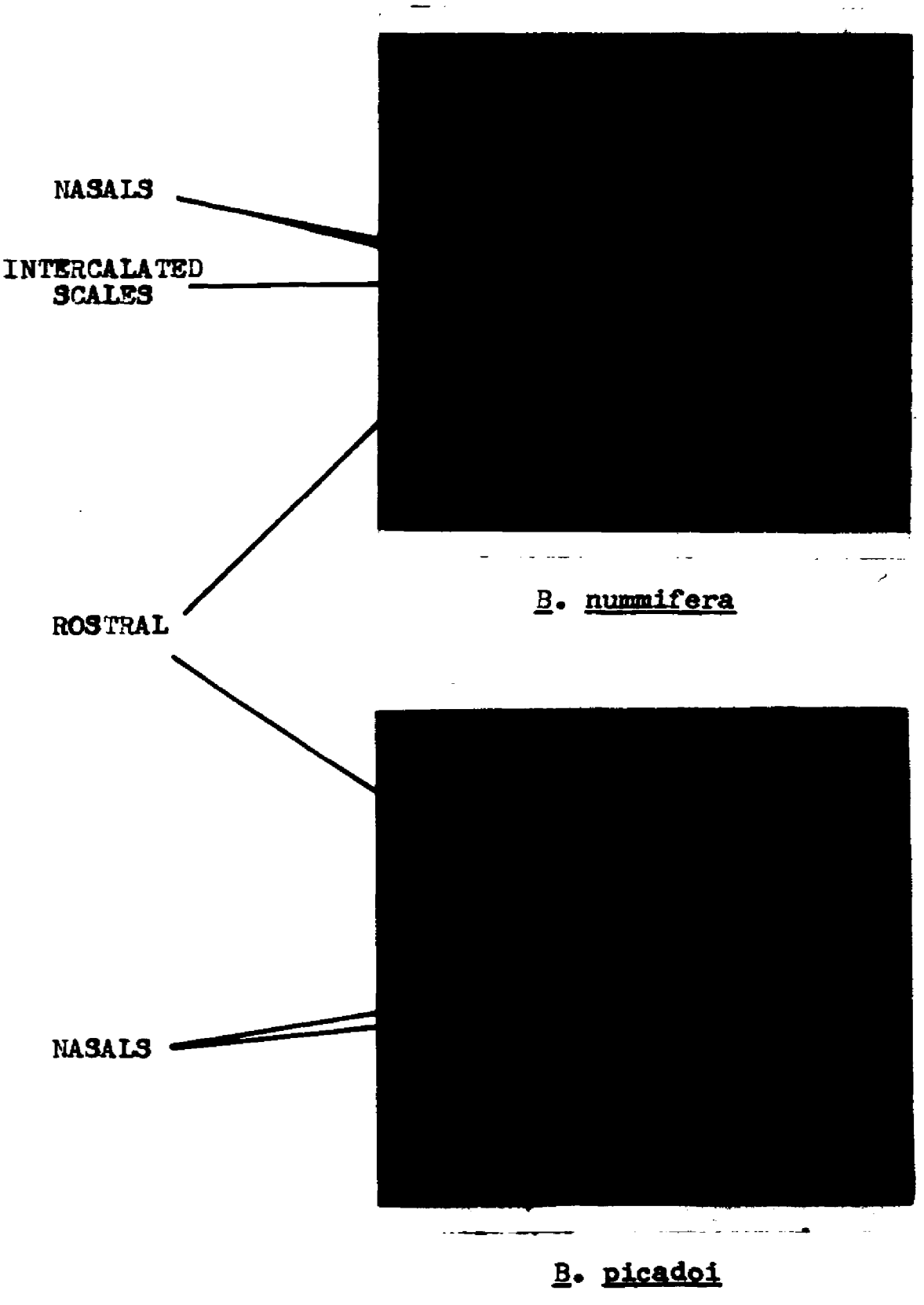


Fig. 6. Distribution of the jumping vipers studied.

• B. nummifera ○ B. picadoi



B. nummifera

B. picadoi

Plate XVI. Morphological differentiation of B. picadoi from B. nummifera.

active specific phosphatase. Among the less active phosphatases, the lowest values were found for deoxyribonuclease and ribonuclease (Appendices VII and VIII).

Electrophoretic Fractionation

A complete absence of cathodal fractions and possession of a large number of anodal bands was the characteristic feature of the electrophoretic pattern of this venom at pH 8.6. L-amino acid dehydrogenase was a very distinct and deeply stained band of moderate mobility. Venom electrophoretic patterns of the four individuals were almost identical (e through h, Plate XVII). Venom pattern of one animal did not vary during the year in which it lived in the serpentarium (a through e, Plate XVII).

Since the venom possessed active phosphatases in spite of the fact that it lacked cathodal bands, on which these activities were found in venoms of other Bothrops, it was important to fractionate the venom and localize the phosphatases along the pattern. Table X shows that specific and unspecific phosphatases were found on the two slowest anodal bands.

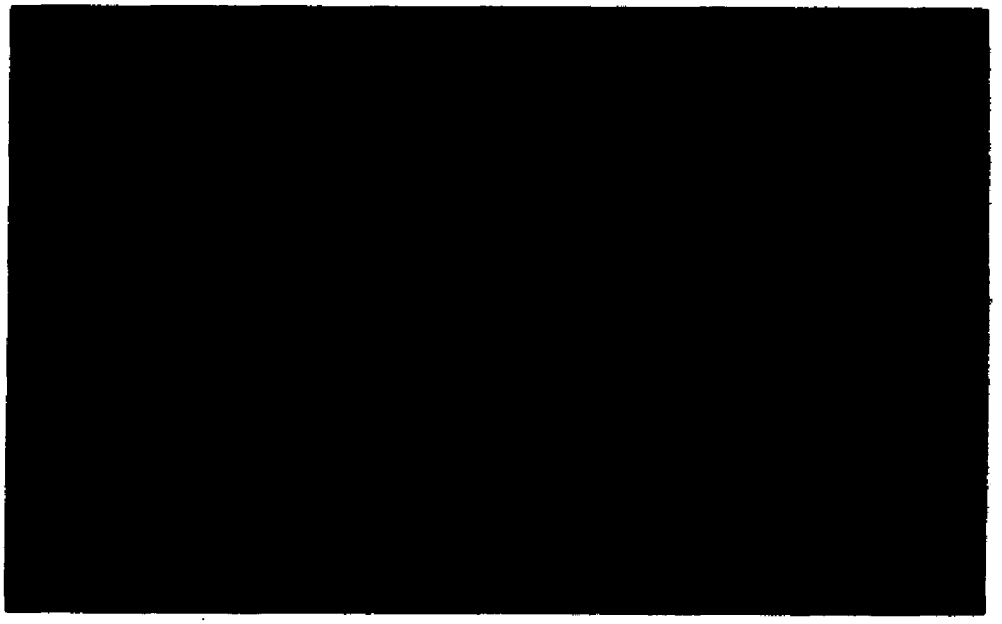


Plate XVII. Starch-gel electrophoretic pattern of B. picadoi venom (diluted 1:2 with 0.85% NaCl).

a through e: Samples taken from one individual during an eight month period and stored frozen for 240, 174, 62, 2 and 0 days.

f, g and h: Samples from three other specimens and kept frozen for 128, 34 and 3 days.

x L-amino acid dehydrogenase

o Phosphatases

TABLE X

LOCALIZATION OF PHOSPHATASE ACTIVITIES ON THE STARCH-GEL ELECTROPHORETIC PATTERN OF BOTHROPS PICADOI VENOM

Phosphatase Activity	Electrophoretic fraction									
	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}
Adenosine-5'-monophosphatase	4+	4+	2+	2+	±	-	-	-	-	-
Nicotinamide-dinucleotidase	4+	4+	±	±	-	-	-	-	-	-
Adenosine-triphosphatase	4+	4+	-	-	-	-	-	-	-	-
Deoxyribonuclease	+	+	-	-	-	-	-	-	-	-
Ribonuclease	+	+	-	-	-	-	-	-	-	-
Unspecific phosphodiesterase	4+	4+	+	±	±	-	-	-	-	-

OTHER LANCE-HEAD SNAKES

Seven additional species of the genus Bothrops occur within Costa Rica. All of these are small snakes. They can be grouped into three types on the basis of morphology and habitat. (1) Palm vipers are arboreal and have a prehensile tail, wide heads and long fangs relative to their size. These animals are dangerous as they have a color pattern that blends with foliage such as that of coffee trees. Coffee pickers are often victims of these snakes. The hands and face are common sites of bites. (2) Hog-nose vipers are terrestrial and recognized by their upturned snouts. (3) The highlands viper is a small terrestrial snake found at high elevations.

Bothrops schlegeli

The horned palm viper or eyelash viper has two or three of several scales above the eye directed upward in the form of spiny horns. This species is also characterized by extreme variation in hue, two color phases being recognized; one is greenish or olive speckled with black and red and the other is lemon or orange yellow (Plate XVIII).

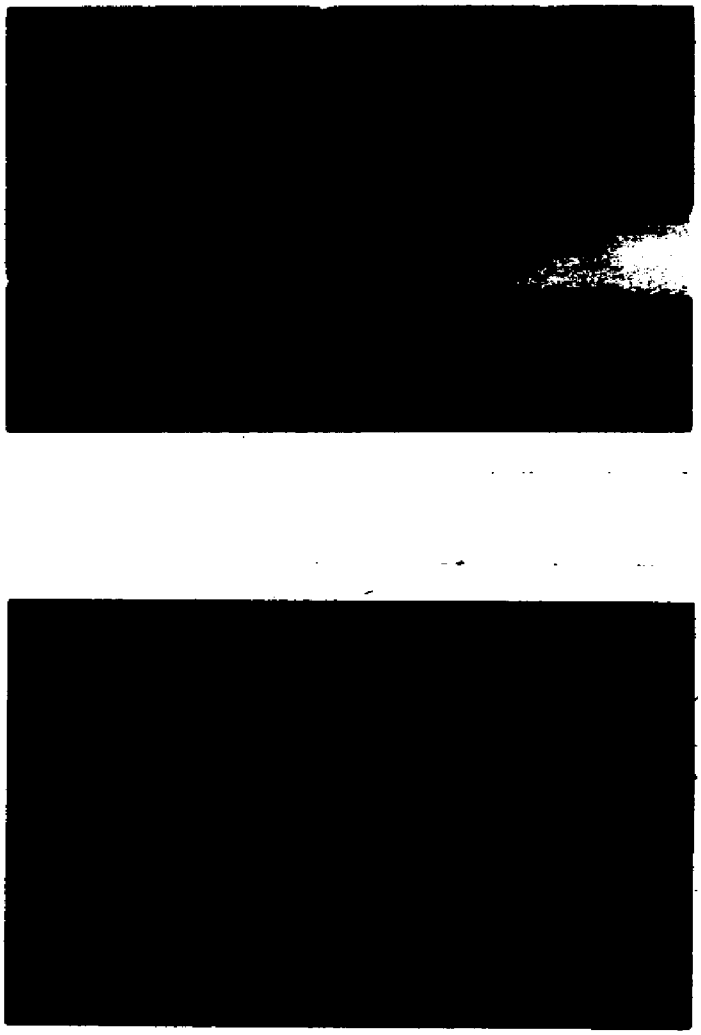


Plate XVIII. Bothrops schlegeli

Above: green phase
Below: yellow phase

Eighty-nine eyelash-vipers were obtained from all ecological zones of Costa Rica, except the North Pacific Zone (Fig. 7).

An indirect hemolysin, comparable in potency to that of B. atrox was found in venom of both color phases. It lacked direct hemolysins and agglutinins. Picado (1931) reported hemolytic and agglutinating properties in venom of this species. He found that venom of the greenish animals hemolysed chicken red blood cells but this property was absent from venom of yellow animals. He conducted some experiments that seemed to prove the presence of neurotoxins in this venom.

Starch-gel electrophoresis was performed on venoms collected from 27 specimens of both color phases of B. schlegeli from the Atlantic and the Pacific Zones. Electrophoretic patterns showed a larger number of anodal than cathodal fractions, but very deeply stained cathodal bands accounted for about 40% of the total proteins. L-amino acid dehydrogenase was a diffuse anodal band of moderate mobility (Plate XIX). The following variations among venom patterns of individual snakes were detected: (1) Slowest anodal band was absent or weak (a and b, Plate XIX) or intense (d, e, f, Plate XIX). (2) Slowest cathodal band was weak (a, c, f, g, Plate XIX) or intense (b, d, e, Plate XIX). (3) Presence of additional very fast anodal fractions in some samples (a, c, f, Plate XIX). (4) Occasional absence of the weak, fastest cathodal band.

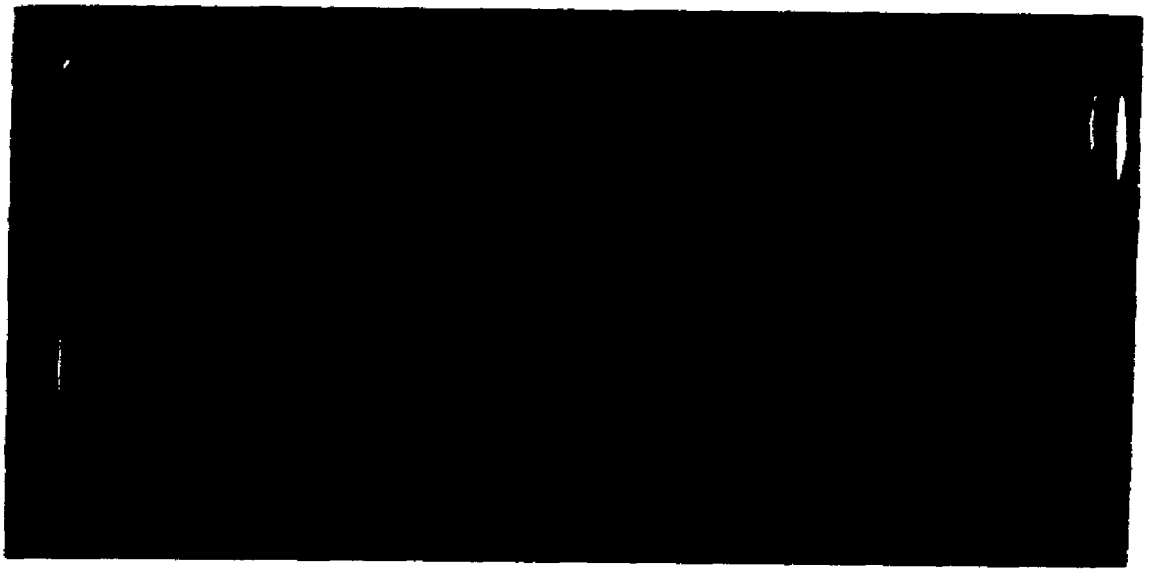


Plate XIX. Starch-gel electrophoretic patterns of venoms of B. schlegeli.

a through d: Atlantic Zone (yellow phase)
f and h: Atlantic Zone (green phase)
e and g: Central Pacific Zone (green phase)

x L-amino acid dehydrogenase.

Although people of Costa Rica commonly consider the yellow and the green phases of the eyelash viper as two different species, the similarity of the electrophoretic patterns of the two types supports their classification in the same species. The variations in patterns were not correlated with either color phase or geographic distribution of the snakes.

Bothrops lateralis

The parrot palm viper is apparently found only in Costa Rica (Ditmars, 1946). Being a close relative of B. schlegeli, it has many of the same characteristics. The lack of spiny horns and its color pattern are distinguishing. It is green above and beneath with a yellow line along each side and yellow spots on the back (Plate XXI). Four specimens were obtained from the Atlantic and Central Pacific Zones (Fig. 7).

Picado (1931) found anticoagulant properties in this venom. From his observation of human accidents, he considered that this venom has very pronounced local action but does not produce systemic effects.

The electrophoretic pattern of this venom was characterized by a large number of anodal fractions and only one weak band of cathodal migration (Plate XXXI). L-amino acid dehydrogenase was a diffuse bright yellow band.

Bothrops nasuta

The nose-horned viper is a representative of the hog-

nosed group of pit vipers. Its snout ends in a proboscis-like appendage. Specimens were obtained from all ecological zones of Costa Rica (Fig. 7).

A mild phosphatidase activity, comparable in potency to that of jumping vipers, was detected in this venom. No direct hemolytic activity was found. Picado (1931) observed its coagulant properties but found that it would not hemolyze human red blood cells. This venom seems to possess a strong local action. Soon after an assistant was bitten on the finger by a very small specimen, milked just a few minutes before the accident, he developed much pain and swelling. Picado (1931) also observed the strong local action of this venom, when one of his associates was bitten.

Electrophoretic patterns of venoms from specimens of B. nasuta from the Atlantic and Pacific Zones showed a predominance of anodal bands. These accounted for over 95% of the total protein of the venom. Only one or two weak cathodal bands were evident. L-amino acid dehydrogenase migrated in a band of moderate velocity, which stained very intensely. Some minor variations were detected in electropherograms of venoms from different specimens (Plate XX).

Bothrops godmani

This highlands viper has an average length of less than two feet and is found in areas of cool climate at elevations over 5,000 feet. The color pattern is pale brown or gray with darker markings. It is a very irritable and

Plate XX. Starch-gel electrophoretic patterns of venoms of B. nasuta.

a-f, h: Atlantic Zone.
g: Central Pacific Zone.
x L-amino acid dehydrogenase.

voracious snake (Plate XXII). Specimens from the Atlantic and Central Pacific Zones were obtained (Fig. 7). Anodal bands far outnumbered cathodal fractions in this venom (Plate XXI).

Plate XXI. Bothrops lateralis

Plate XXII. Bothrops godmani

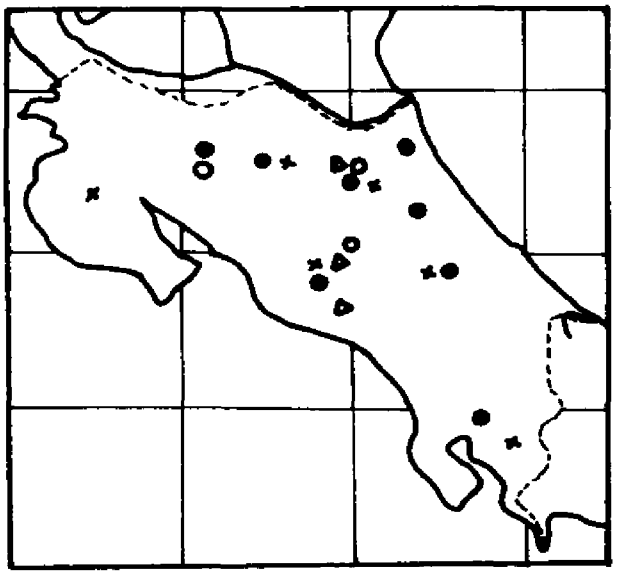


Fig. 7. Distribution of the small lance-head vipers studied.

- ● B. schlegeli
- o B. lateralis
- x B. nasuta
- ▲ B. godmani

OTHER CROTALIDAE

Only two species of pit vipers that are not lance-heads are found south of Mexico. One of these is the rattlesnake, Cpgtalus durissus; the other is the famous bushmaster, Lachesis muta.

Lachesis muta

The bushmaster is the only species of its genus. It is the largest viper in the world, reaching a length of 12 feet and the only American viper that lays eggs (Ditmars, 1946). Morphologically it is more closely related to rattlesnakes than to lance-heads. Although its range extends from Costa Rica through Brazil, it is more frequently found in Costa Rica and Panama. Its ground color is a pale brown. A series of dark brown or black blotches occur along its body, wide on the back and narrow on the sides. The pattern of blotches is quite similar to that of the jumping viper and the reverse of the fer-de-lance. The bushmaster vibrates its rattleless tail in a manner similar to rattlesnakes. For this reason it is called the mute rattler in Central America (Plate XXIII). This snake has been con-

Plate XXIII. Lachesis muta

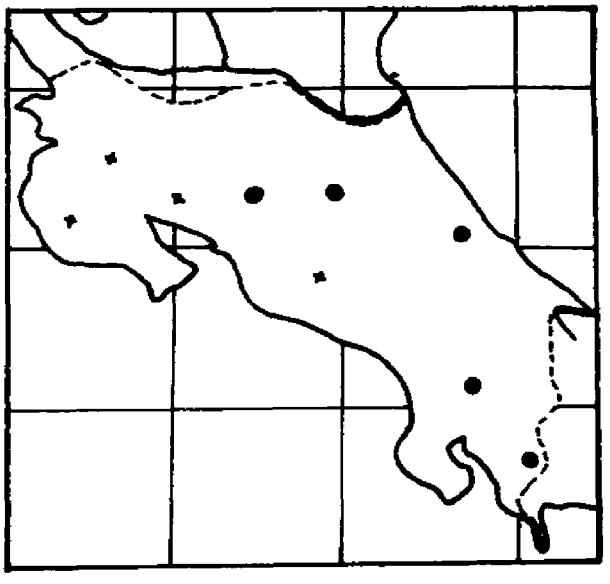


Fig. 8. Distribution of other crotalid snakes studied.

• Lachesis muta

x Crotalus durissus

sidered as confined to the South Pacific Zone of Costa Rica. However, most of the specimens obtained were captured in several localities of the Atlantic Zone (Fig. 8).

The venom electropherogram is characterized by absence of cathodal bands and possession of a distinct yellow band of mobility similar to that of Bothrops L-amino acid dehydrogenases (Plate XXXI). According to Picado (1931) L. muta venom is highly hemolytic and exhibits the most pronounced agglutinating power of all venoms of Costa Rican snakes. He reported also that this venom possesses a strong local action. Envenomation by the huge bushmaster can not be counteracted by the so-called polyvalent sera prepared with venoms of genera Bothrops and Crotalus (Picado, 1931; Bücherl, 1961).

Crotalus durissus

The "cascabela" is the only species of rattlesnake found in Costa Rica. It possesses a diamondback design but has elongate bands on its neck. These are heavy bodied animals with a ridge on their backs and with a head that is proportionately small (Plate XXIV). This species is restricted to the drier areas of Costa Rica, in the North and Central Pacific Zones (Fig. 8). Forty big animals, most of them captured in the North Pacific Zone (Province of Guanacaste), were studied. Work was carried out also with venoms from twenty-three youngs born in the serpentarium.

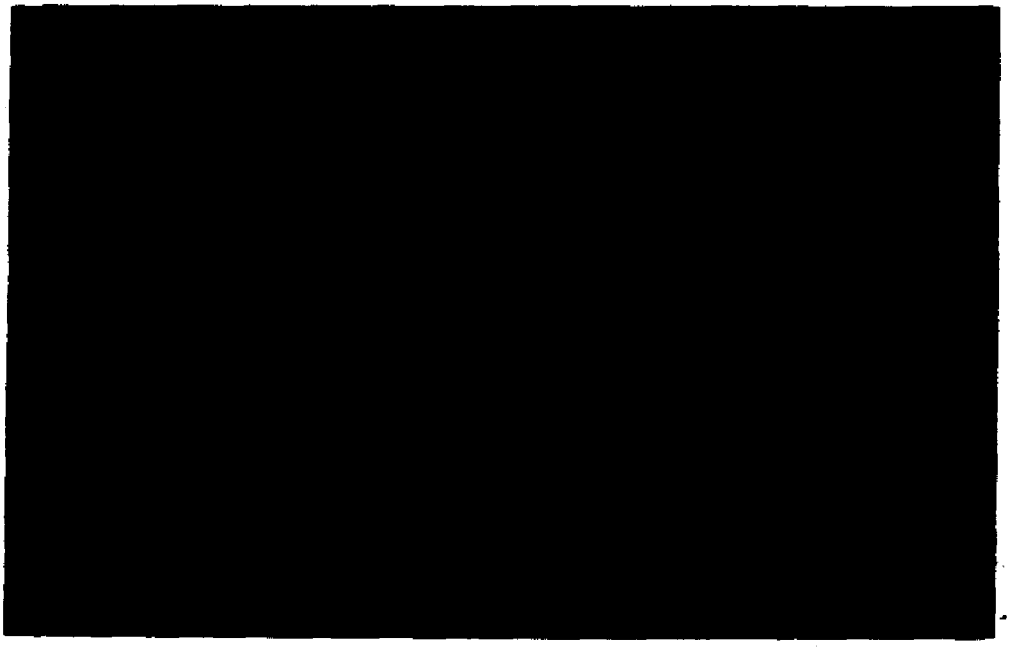


Plate XXIV. Big specimen of Crotalus durissus.

Gross Composition of Venom

About one fourth of this venom was solid, of which proteins accounted for 75%. pH was in the neighborhood of 5.7 Average sodium concentration was around 200 mEq/L, whereas potassium level was below 10 mEq/L. Average chloride concentration was 25 mEq/L. Phosphate was absent (Appendix II).

Effects on Blood Clotting and Action on Human Erythrocytes

This venom exhibited two actions on clotting of human plasma and bovine fibrinogen: a clot promoting action at high dilutions due to a thrombin-like substance and an inhibitory effect of fibrinolytic nature at high concentrations of venom. It contained no prothrombinase activity (Appendices III, IV, and V). Picado (1931) also found in this venom the double character of delaying coagulation when added to rabbit blood and of producing clotting of citrated blood. Eagle (1937) reported on the coagulant action of venom of the Brazilian subspecies, Crotalus terrificus (= C. durissus terrificus; Klauber, 1956); anticoagulant properties have also been found in venom of the South American rattlesnake (Klobusitzky, 1961).

C. durissus venom possessed a mild indirect hemolysin for human erythrocytes but it lacked direct hemolysins and agglutinins (Appendices VII and IX). Within the usual range of venom dilutions used to measure hemolytic activity in

western diamondback or fer-de-lance venoms (1:400,000 to 1:4,000,000) no hemolysis occurs with venom of C. durissus. This low activity explains why some investigators considered that C. durissus venom contained no hemolysins for human erythrocytes (Picado, 1931).

Other Enzyme Activities

Proteolytic enzymes of the tryptic, chymotryptic, rennin and papain types were found in C. durissus venom, but pepsin, cathepsin, acid and alkaline phosphomonoesterases, catalase and cholinesterase were absent (Appendices VI and IX). Adenosine-5'-monophosphatase was the most active specific phosphatase. Nicotinamide dinucleotidase and adenosine-triphosphatase activities were about 10 times lower than adenosine-5'-monophosphatase. Ribonuclease and deoxyribonuclease activities were very feeble (Appendix VIII).

Picado (1931) early emphasized the geographic variation in site of toxic action of venoms of the Neotropical rattlesnake. The South American subspecies, C. durissus terrificus, produces venoms with strong neurotoxic action and almost no local effects. The Mexican subspecies, C. durissus culminatus, like species of Crotalus found in the United States, produces venoms with intense local effects but no neurotoxic actions (Van Heyningen, 1954). Toxic action of the Costa Rican subspecies, C. durissus durissus, combines neurotoxic and local effects.

Electrophoretic Fractionation

The electrophoretic pattern of C. durissus venom was characterized by a predominance of anodal fractions (7 bands) over cathodal fractions (2 bands) Plate XXV).

Venom from one specimen from the North Pacific Zone was fractionated to localize enzyme activities and toxicity of bands along the pattern (Tables XI and XII). All phosphatases were found in a weak band, the slowest among those migrating toward the cathode. The yellow L-amino acid dehydrogenase was the slowest anodal fraction, and again phosphatidase was of weak concentration and one of the fastest anodal bands. All proteolytic enzymes, including fibrinolytic activity, migrated in a concentrated band; the fastest cathodal band of the pattern. Thrombin-like activity was localized in a broad diffuse anodal band, which was highly toxic.

Crotoxin, a protein responsible for the toxocity of venom of the South American rattlesnake, is a neurotoxin and comprises 60% of the total protein of the venom. It was isolated and crystallyzed by salt fractionation methods (Slotta and Fraenkel-Conrat, 1938, 1939). Although crotoxin was homogeneous by solubility, electrophoretic and ultracentrifugal criteria (Gralén and Svedberg, 1938; Slotta and Fraenkel-Conrat, 1939), it exhibited also phosphatidase activity. Fraenkel-Conrat and Singer (1956) separated crotoxin into two fractions by means of the fluoro-dinitro-

Plate XXV. Starch-gel electrophoretic pattern of venom from one specimen of Crotalus durissus.

TABLE XI

ENZYME ACTIVITIES AND TOXICITY
OF ELECTROPHORETIC FRACTIONS
OF VENOM OF ONE SPECIMEN
OF CROTALUS DURISSUS

Activity	Electrophoretic fraction								
	C_2	C_1	A_1	A_2	A_3	A_4	A_5	A_6	A_7
Phosphatases	-	4+	2+	-	-	-	-	-	-
L-amino acid dehydrogenase	-	-	+	+	-	-	-	-	-
Phosphatidase A	-	-	-	-	-	-	±	4+	-
Trypsin	4+	1+	-	-	-	-	-	-	-
Chymotrypsin	4+	-	-	-	-	-	-	-	-
Papain	4+	-	-	-	-	-	-	-	-
Rennin	4+	-	-	-	-	-	-	-	-
Thrombin	-	±	±	±	-	-	4+	3+	±
Fibrinolysin	4+	2+	±	±	-	-	-	-	-
Toxicity	2+	2+	1+	1+	3+	3+	4+	2+	1+

TABLE XII

TOXICITY OF ELECTROPHORETIC FRACTIONS
OF VENOM FROM ONE SPECIMEN
OF CROTALUS DURISSUS

Fraction	No. mice injected	% mortality	Survival time (hours)	Toxicity
C_2	4	50	3-24	2+
C_1	8	50	3-24	2+
A_1	7	14	12-24	1+
A_2	5	20	12-24	1+
A_3	8	88	5-48	3+
A_4	7	86	5-48	3+
A_5	20	95	4-12	4+
A_6	15	53	17-24	2+
A_7	3	33	12-36	1+

benzene reaction. They concluded that crotoxin was a firm complex between an acidic and a basic protein. However, since this means of fractionation destroys all activities, they were unable to identify the components. Neumann (1955) separated crotoxin into a neurotoxin and a non-toxic phosphatidase by chromatography. Neumann named the toxic fraction crotactin and studied its biochemical and pharmacological properties extensively (Neumann and Habermann, 1955; Habermann and Neumann, 1956).

Of the electrophoretic fractions separated on the starch-gel from venom of the Central American rattlesnake subspecies, fraction A_5 had a close resemblance to crotactin. This fraction comprised about a fourth of the total venom and exhibited the highest toxicity of any fraction. Like crotactin, fraction A_5 was phosphatidase free. However, the proximity of fraction A_5 to the phosphatidase contained in fraction A_6 suggests that these two activities might be difficult to separate. Fraction A_5 differred from crotactin in exhibiting strong thrombic activity.

The fact that proteolytic and fibrinolytic activities were located in a cathodal band well resolved from the clot promoting action contradicts the common hypothesis that coagulant properties of <u>all</u> venoms are due to their proteolytic activities (Eagle, 1937; Jánszky, 1950).

<u>Geographic Variations</u>

Although no differences were found in the frequency of

occurrence of the various electrophoretic fractions among populations from the North and Central Pacific Zones of Costa Rica, other workers have observed geographic variation in venom proteins within this species. Gonçalves and coworkers found differences among venoms produced by the South American race of this species in Brazil and Argentina (Gonçalves and Vieira, 1950, 1956; Gonçalves, 1956; Gonçalves, and Deutsch, 1956). A neurotoxin of cathodal migration, which they named crotamine, was present in venoms of species from southern Brazil and Argentina but absent in venoms from northern and central Brazil. They also observed that venom of northern specimens contained L-amino acid dehydrogenase but southern specimens lacked this enzyme. Schenberg (1959) made a thorough sampling in the area of intergradation between northern and southern forms. In that region three types of specimens coexisted: (1) northern type with L-amino acid dehydrogenase but no crotamine, (2) southern type with crotamine but no L-amino acid dehydrogenase, and (3) hybrid types with other combinations of these proteins, e.g. with crotamine and L-amino acid dehydrogenase.

Individual Variations

Starch-gel electrophoresis was applied to rattlesnake venoms of 15 adult specimens collected in the North and Central Pacific Zones. A few variations were observed among the electrophoretic patterns of different specimens (Plate XXVI). For example, some samples showed an additional,

Plate XXVI. Starch-gel electrophoretic patterns of venoms from 8 specimens of C. durissus.

a through d: from Northern Pacific zone.
e through h: from Central Pacific zone.

intense anodal band that migrated faster than A_7. Bands A_6 and A_7 were weak in some individuals but intense in others. The fastest cathodal band, usually the most deeply stained fraction, was moderately stained or even very weak in some individuals. One individual (sample f, Plate XXVI) showed an extra cathodal fraction between the phosphatase and the protease bands.

Developmental Variations

A litter of 23 newborn rattlesnakes were milked two months after birth and a second time, one month later. Seventeen produced colorless venom at both milkings; two who produced colorless venoms at the first milking secreted slightly yellow samples at the second milking. Four had light yellow samples in both milkings. Colorless venoms were never observed in the 40 adult rattlesnakes studied.

Colorless or slightly yellow samples were subjected to the same experiments as colorless venoms from B. nummifera. Solid and protein contents as well as osmotic pressure were lower than the minimum values found in yellow samples from adult specimens (Table XIII). L-amino acid dehydrogenase activity was very low and did not increase significantly after the addition of flavin adenine dinucleotide to the assay mixture. An electrophoretic pattern of one colorless specimen showed almost absence of bands in the region in which L-amino acid dehydrogenase normally was found. Band A_3 and the fastest cathodal band (C_2) were also absent.

TABLE XIII

STUDY OF COLORLESS AND SLIGHTLY YELLOW VENOMS OF CROTALUS DURISSUS

	No. Animals	Newborn	Adult (yellow)
Solid content (g/100 ml)	13	15.8±2.9[a] (12.3-20.8)[b]	26.9±4.2 (16.4 -35.6)
Protein content (g/100 ml)	7	6.5±2.1 (3.8-10.1)	22.0±3.6 (11.1-27.0)
Osmotic pressure (m. Osm/L)	5	164±31 (128-213)	343±20 (300-375)

[a]Avg. ± S.D. [b]Range

The highly toxic fraction (A_5) was present in venom from this newborn snake (Plate XXVII). Newborn rattlesnakes producing colorless venoms were able to kill mice in a short time. Several slightly yellow samples showed patterns that were almost identical to those of adults, except that L-amino acid dehydrogenase appeared to be fractionated into several bands of different intensity or to be a single, sharp band (Plate XXVIII).

Plate XXVII. Starch-gel electrophoretic patterns of rattlesnake venoms.

Above: yellow venom from an adult specimen.
Below: colorless venom from a newborn animal.

Plate XXVIII. Starch-gel electrophoretic patterns of slightly yellow venoms from 6 newborn rattlesnakes.

CORAL SNAKES (ELAPIDAE)

The coral snakes are the only New World representatives of family Elapidae. They differ from cobras, their relatives, in their small slender body and in the lack of striking ability. They are narrow headed, with very pretty pattern and look anything but dangerous. Micrurus nigrocinctus, the most common venomous coral in Costa Rica, is characterized by the sequence of black, yellow and red rings shown in Plate XXIX. Forty-three specimens were obtained from many localities, including the suburbs of the capital, in all ecological zones of the country (Fig. 9).

Venoms produced by corals and cobras have the common property of being very neurotoxic. Coral venom has been reported as containing an even more potent neurotoxin than cobra venom (Phisalix, 1922; Picado, 1931; Van Heyningen, 1954).

Simultaneous assays on lyophilized venoms of Micrurus nigrocinctus and Naja naja (spectacled cobra), for purposes of comparison, showed that both venoms possessed strong direct and indirect hemolysins. However, these venoms were unable to agglutinate human erythrocytes. Coral venom did not exhibit acetylcholinesterase activity, whereas cobra venom

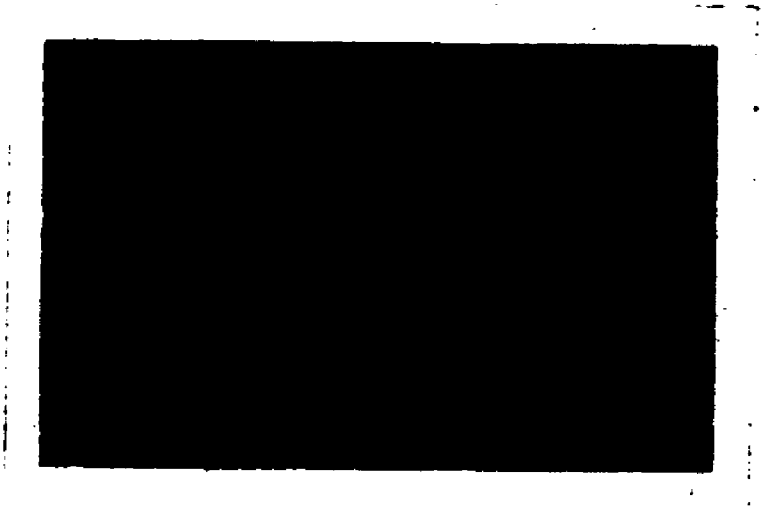


Plate XXIX. Micrurus nigrocinctus

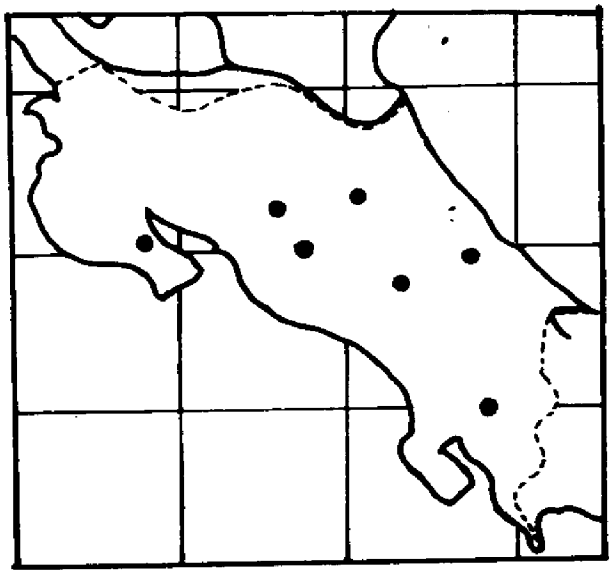


Fig. 9. Distribution of M. nigrocinctus studied.

is one of the richest sources of that enzyme (Iyengar, et al., 1938; Zeller, 1951).

Anticoagulant and hemolytic properties were found by other workers in M. nigrocinctus venom (Picado, 1931; Klobusitzky, 1961).

Coral and cobra venoms showed a predominance of cathodal bands on starch-gel electropherograms (Plate XXX). Positions of electrodes had to be reversed to prevent loss of cathodal fractions. These findings were agreeable with previous results of paper electrophoresis on venom of Naja tripudians (Grassman and Hannig, 1954).

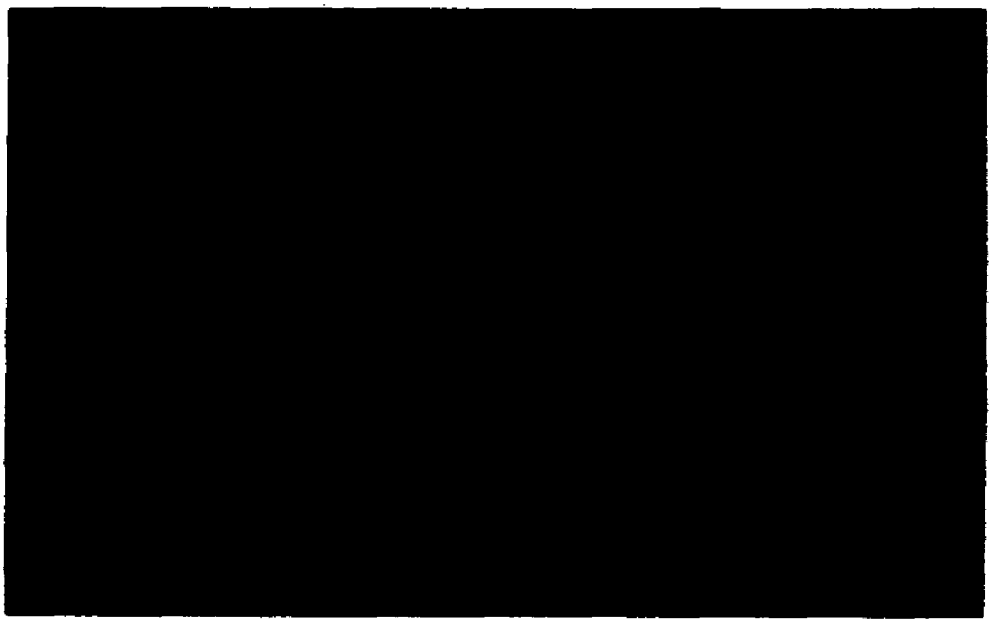


Plate XXX. Starch-gel electrophoretic patterns of two elapid venoms.

a. Naja naja (spectacled cobra)
b. Micrurus nigrocinctus (coral)

CHEMICAL TAXONOMY OF VENOMOUS SNAKES

For many years scientists have depended largely upon morphological characteristics to identify organisms, estimate their relationships and follow their evolution. Biochemistry offers the taxonomist an ever-enlarging battery of techniques which can be used to supplement classical methods. As morphological variation is the result of underlying biochemical differences between organisms, the comparison of biochemical similarities and differences should give more sensitive indications of relationships than gross morphology. Comparisons of blood, hemolymph and egg white proteins have already demonstrated the usefulness of chemical characteristics in taxonomy. These are reviewed by Sibley (1960), Engle and Woods (1960) and Dessauer and Fox (1963). Venoms have promise of being useful tissue fluids in solving taxonomic problems.

Developmental and Physiological Variations

To properly evaluate the taxonomic significance of chemical characteristics, effects of developmental and physiological variables must be recognized (Dessauer and Fox, 1963). Developmental changes have been noted in properties

of proteins of many vertebrates and invertebrates. Developmental factors also must be considered in evaluating the taxonomic significance of components of snake venoms. Newborn Crotalus durissus and Bothrops nummifera lack L-amino acid dehydrogenase and other venom enzymes which are present in older snakes. Physiological effects may also be important as certain adult jumping vipers also lack L-amino acid dehydrogenase in their venoms. On the basis of studies to date, however, the cause of the lack of this dehydrogenase is unknown. The absence of this enzyme may be genetically determined as is the case in the southernmost race of Crotalus durissus (Schenberg, 1959).

Intraspecific Variations

High resolution techniques of protein fractionation allow for discriminating analysis of components of various tissues. In some cases the number of differences observed between samples of tissue from various individuals is so extensive that each animal can be characterized as a "biochemical individual" (Medawar, 1958). In other cases the frequency of occurrence of specific proteins characterizes a population (Dobzhansky, 1951).

Individual Variations. Venom proteins appear to be more variable in electrophoretic properties than either egg white protein or blood proteins (Dessauer, personal communication). Certain individuals from single populations of B.

atrox of restricted geographic areas could be identified by differences in the number, migration and quantity of proteins of their starch-gel electrophoretic patterns. On occasion patterns were very individualistic. For example, only one animal of 46 fer-de-lances secreted a highly concentrated, cathodal band of fast migration rate (Plate VIII). Each venom pattern of eight fer-de-lances shown in Plate VII was different. Such variations were not correlated with size, duration of captivity, feeding, time of milking or duration of venom storage before analysis.

Geographic Variations. Two striking examples of variations in venom proteins between geographically separated populations were observed. Populations of Bothrops nummifera captured in the Atlantic Zone could be distinguished from populations from the Pacific Zones by their proteolytic enzymes. Proteolytic activities migrated in a single band in this species, but the mobilities were distinctly different in animals from the two zones. The band of cathodal migration was restricted to Pacific Zone animals; the anodal band was restricted to Atlantic Zone animals. Although no single venom protein was restricted to specific populations of fer-de-lance, populations from the Atlantic, Central Pacific, and South Pacific Zones varied in the frequency of occurrence of different polymorphic forms of L-amino acid dehydrogenase, trypsin and other enzymes. Protein variations in venoms among populations of the South American rattlesnake, dis-

cussed in Chapter VII, are similar to variations in B. atrox and B. nummifera.

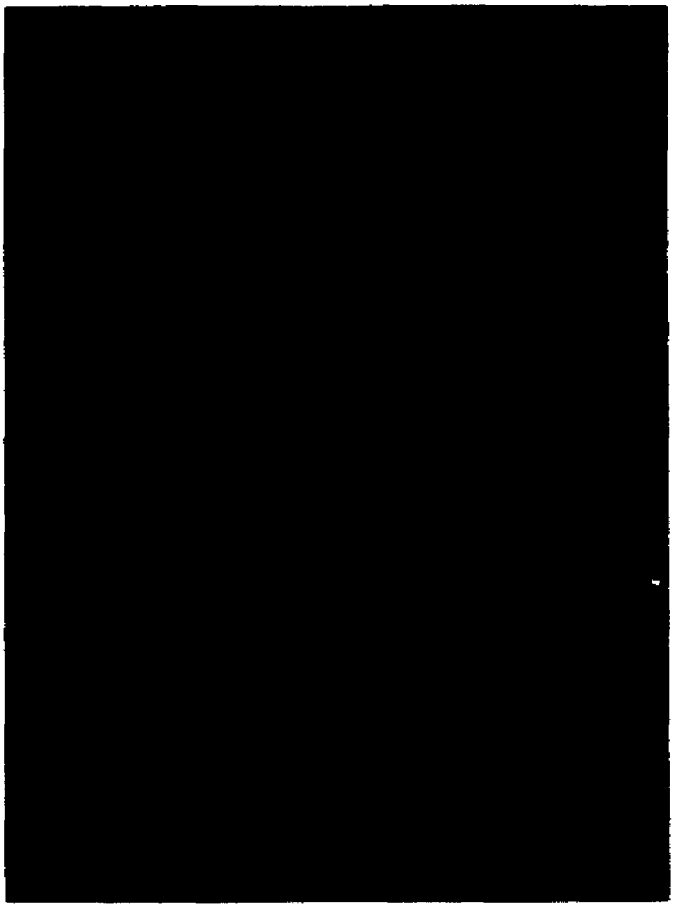
Reproductive isolation, caused by mountain barriers, probably is responsible for the evolution of these venom differences. Morphological studies have shown that topographic isolation commonly is more important than other ecological barriers in contributing to evolutionary changes (Allee, et al., 1950). Research on intraspecific variants separated by topographic barriers has been performed quite recently by other investigators. Two subspecies of the whiptail lizard Cnemidophorus tigris, recognized on morphological characteristics, are isolated by mountains of a maximal elevation of about 5,700 feet in a restricted geographical area between Arizona and New Mexico. The only hybridizing populations are found close to mountain passes through which the western subspecies penetrates the uplands to hybridize with the other subspecies on the eastern side of the mountains (Zweifel, 1962). By using starch-gel electrophoresis, Dessauer, et al.,(1962b) found that plasma protein patterns correlated well with morphology at both ends of the hybridization zone and that there was a gradual shift in variable proteins within this zone. Minor differences could be detected more precisely by biochemical techniques than by morphological observations.

Schenberg (1963) studied a large number of specimens of 6 subspecies of Bothrops neuwiedi collected in different

areas of Brazil. He recognized 20 types of venom in this species on the basis of immunological composition. He emphasized that these differences were not artifacts since venom obtained from a large number of extractions from the same individual was constant in its immune properties. Differences in climate of the region of origin, season, diet, and time in captivity did not cause venom variations. Venom patterns among snakes from the same area were very similar. He even utilized the consistency between geographic origin of the snakes and venom properties to identify the place of capture of the animals. On the basis of the above considerations, he concluded that the variations were genetically determined. He believes that regional differences in composition of snake venom may be the cause of some failures in treatment of snakebite with immune sera.

Variations between Species of the Same Genus

Seven species of Bothrops could be distinguished from each other by the electrophoretic patterns of their venom proteins (Plate XXI). For example, although individual variations occurred, the over-all characteristics of patterns were uniform enough to allow identification of B. atrox. Populations from the Atlantic and Pacific Zones could be distinguished on the basis of the frequency of certain venom proteins (Chap. IV), but animals from these ecological zones were not different species since the "variable proteins" were present in populations of each zone.



B. atrox
B. nummifera (Pacific Zone)
B. nummifera (Atlantic Zone)
B. picadoi
B. nasuta
B. godmani
B. schlegeli (yellow phase)
B. schlegeli (green phase)
B. lateralis
C. durissus
L. muta
C. atrox

Plate XXXI. Starch-gel electrophoretic patterns of venoms of ten crotalid snakes.

x L-amino acid dehydrogenase

Bothrops nummifera is a special case. Snakes from the Atlantic Zone could be distinguished from those from the Pacific Zones; both forms could be distinguished from other Bothrops. Are the jumping vipers from different ecological zones different species? Although critical intergrades have not been found as yet, I believe, for the present, that the two forms should be classified within the same species. Except for the proteolytic fractions, the electropherograms of venoms from the two zones were identical. Venoms from both forms contained high concentrations of potassium and low sodium. This is unique among Bothrops studied. Also, both forms had low specific and unspecific phosphatases; in contrast, these activities were very high in B. atrox and B. picadoi, the only other Bothrops in which phosphatase activities were assayed.

Bothrops picadoi has only recently been separated from B. nummifera as a distinct species (Dunn, 1939; see Taylor, 1954). As was shown in Plate XVI, this species is distinguished from B. nummifera by a simple scale difference. Venom electrophoretic patterns, however, clearly distinguish the two species (Plates XII, XV and XXXI). Yet, it would appear from venom characteristics that B. picadoi is related to B. nummifera, especially to sympatric populations from the Atlantic Zone. Mobilities of L-amino acid dehydrogenases of the two species were identical. Sodium was high and potassium low in this species, but certain individuals of B. nummifera from the Atlantic Zone also

had relatively high sodium and low potassium (Fig. 5).

The two species of Crotalus studied were clearly distinguished by chemical differences of their venoms. C. atrox venom possessed only anticoagulant activity whereas C. durissus venom contained both coagulant and anticoagulant fractions. Both species contained indirect hemolysins, but the hemolysin of the western diamondback rattlesnake was much more active than that of the Central American form. C. durissus venom is neurotoxic but C. atrox is not neurotoxic (Picado, 1931; Van Heyningen, 1954). Electrophoretic properties were distinctly different. For example, proteolytic activity was in a cathodal band in C. durissus (Table XII) and in an anodal band in C. atrox (Jiménez-Porras, 1961).

Comparison of Higher Taxonomic Categories

Certain properties of venoms serve to distinguish families of snakes and to illustrate similarities of proteins within each family.

Venoms of species of three genera of Crotalidae were compared. These venoms contained about 25% of solids, of which three-fourths were proteins. Sodium usually was the predominate cation (exception, B. nummifera); chloride was low and inorganic phosphate was absent. Proteins probably were the predominate anions as the electrophoretic pattern commonly showed a great predominance of anodal fractions (Plate XXXI). pH was around 5.8.

Venoms of Crotalidae cause localized actions on snakebite victims. This is largely due to the presence of potent proteolytic enzymes of trypsin, chymotrypsin, rennin and papain-like properties. Peptic and catheptic activities were lacking. The richest source of L-amino acid dehydrogenase is probably the venom of crotalid snakes (Zeller, 1951). This enzyme, almost non-toxic when pure, probably acts synergistically with proteases in digesting tissues of snakebite victims. Fractions possessing proteolytic activities and L-amino acid dehydrogenase were among the most concentrated proteins of crotalid venoms (Plate XXXI). L-amino acid dehydrogenases of Bothrops (seven species) and Lachesis migrated much faster than those of Crotalus (two species). This enzyme was of anodal nature in all venoms.

Clot promoting fractions (thrombin) were present in most species (absent in C. atrox). Fibrinolytic activity, migrating at the same rate as proteolytic enzymes, was found in all crotalid venoms.

Indirect hemolysins, which always migrated electrophoretically in rapid anodal fractions, were very active in some species (B. atrox, B. schlegeli, and C. atrox) and very weak in others (B. nummifera, B. picadoi, B. nasuta and C. durissus). Direct hemolysins almost always were absent. Specific phosphatases and unspecific phosphodiesterase of slow migration at pH 8.6 were present in all species studied (Appendix VIII). Acid and alkaline phos-

phomonoesterases were absent from these venoms. Hyaluronidase was present.

Venoms of certain species of Crotalidae cause systemic actions as well as localized actions on snakebite victims. For example, venoms of snakes of genus Agkistrodon and of the Central and South American rattlesnake, Crotalus durissus, have both local and neurotoxic properties (Picado, 1931; Van Heyningen, 1954; Minton, 1956; Plagnol and Martin, 1957). Crotactin and crotamine are the main substances responsible for neurotoxicity in C. durissus (Neumann, 1955; Gonçalves, 1956). Acetylcholinesterase is absent from these species as well as from other Crotalidae.

Venoms of Elapidae have no local action but are strongly neurotoxic (Phisalix, 1922; Picado, 1931; Van Heyningen, 1954). Acetylcholinesterase, present in most elapid venoms (e.g., cobra), was suspected as being the cause of their neurotoxicity (Iyengar, et al., 1938); however, venom of Micrurus nigrocinctus (coral snake) lacks this enzyme, yet this venom is highly neurotoxic. Both direct and indirect hemolysins are highly active in elapid venoms. Some of these venoms contain relatively high amounts of L-amino acid dehydrogenase (coral snake); others contain low amounts (cobra). Most elapid venoms (cobra, tiger snake, corals) inhibit blood clotting (Picado, 1931; Devi, et al., 1956; Klobusitzky, 1959, 1961). However, venom of M. mipartitus, a coral snake, is coagulant as venoms of viperine snakes (Picado, 1931). Electrophoretic fractions of elapid venoms

are predominately cathodal (Plate XXX).

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APPENDIX I

SIZE OF SNAKES AND VENOM YIELDS

Species	No. of Specimens	Weight kg	Length m	Avg. captivity months	Milkings No./ animal	ml/ sample
B. atrox	116	0.7[a] (0.2-4.3)[b]	1.2 (0.8-2)	3.2 (1-11)	3.5 (1-13)	0.85 (0.1-6.2)
B. nummifera (adults)	12	0.7 (0.15-1.1)	0.8 (0.5-1)	3.5 (2-6)	5 (2-9)	0.6 (0.2-1.4)
B. nummifera (young and newborn)	34	0.03 (0.02-0.12)	20 (0.2-0.5)	2 (0.5-3.5)	1.5 (1-3)	0.09 (0.05-0.1)
B. picadoi	4	1.1 (0.8-1.6)	1	6 (3-13)	6.5 (3-13)	0.75 (0.2-1.5)
B. schlegeli	89	0.07 (0.02-0.15)	0.51 (0.3-0.7)	4 (1-9)	4 (1-12)	0.15 (0.05-0.3)
B. lateralis	4	0.09 (0.06-0.12)	0.60 (0.4-0.8)	3 (1-6)	1.7 (1-3)	0.07 (0.05-0.1)
B. nasuta	67	0.09 (0.03-0.21)	0.48 (0.3-0.6)	5.5 (1-10)	6 (2-11)	0.25 (0.1-0.4)
B. godmani	6	0.06 (0.03-0.09)	0.50 (0.4-0.6)	10 (4-14)	3 (1-6)	0.12 (0.05-0.2)

[a] Avg. [b] Range

APPENDIX I (Contd.)

SIZE OF SNAKES AND VENOM YIELDS

	No. of Specimens	Weight kg	Length m	Avg. captivity months	Milkings No./animal	Milkings ml/sample
C. durissus (adults)	40	1.6[a] (0.6-3.1)[b]	1.4 (1-1.7)	4 (2-7)	5 (1-9)	0.7 (0.3-1.5)
C. durissus (newborn)	23	0.07 (0.06-0.09)	0.47 (0.4-0.5)	3 (1-4.5)	2 (1-3)	0.09 (0.05-0.2)
L. muta	6	2.8 (0.4-5.0)	1.7 (1-2.2)	2.6 (2-4)	2	0.6 (0.2-1.0)
M. nigrocinctus	43	0.04 (0.02-0.06)	0.50 (0.2-0.7)	2 1-4	2 (1-3)	0.05 (0.03-0.8)

[a] Avg. [b] Range

APPENDIX II

GROSS COMPOSITION OF VENOMS

Solids g/100 ml	Protein g/100 ml	Osm. pressure m osm/L	pH	Sodium mEq/l	Potassium mEq/L	Chloride mEq/L
			B. atrox			
28.5 ± 4.2[a]	23.5 ± 4.4	339 ± 28	5.80 ± 0.15	179 ± 17.5	17 ± 11.3	18.5 ± 6.2
20.0 - 43.0[b]	14.7 - 36.0	290 - 400	5.60 - 6.18	140 - 208	4.4 - 54	8.0 - 35
67[c]	53	34	34	30	30	28
			B. nummifera			
19.9 ± 3.8	14.0 ± 3.4	322 ± 12	5.86 ± 0.14	49 ± 24	147 ± 18.2	5.2 ± 2.2
15.4 - 27.8	10.3 - 20.9	250 - 396	5.57 - 6.05	11 - 166	50 - 284	2.8 - 9.6
10	10	17	17	25	25	10
			B. picadoi			
21.3 ± 2.0	16.2 ± 2.0	308 ± 3.3	5.69 ± 0.09	189 ± 23	10.7 ± 2.2	2.9 ± 0.8
18.4 - 24.6	13.1 - 19.3	268 - 365	5.60 - 5.80	159 - 234	8.8 - 15.2	2.5 - 5.0
10	10	10	10	10	10	10
			C. durissus			
26.9 ± 4.2	22.0 ± 3.6	343 ± 20	5.69 ± 0.15	204 ± 16	8.2 ± 1.8	25.4 ± 8.3
16.4 - 35.6	11.1 - 27.0	300 - 375	5.30 - 5.90	164 - 230	5.8 - 11.8	12.5 - 43.3
40	40	20	20	20	20	20

[a]Avg. ± S.D. [b]Range [c]No. of samples

APPENDIX III

ACTION ON CLOTTING OF RECALCIFIED PLASMA

Venom Dilution	*B. atrox*	*B. nummifera*	*C. durissus*
1 : 10	Prevented	Prevented	Prevented
1 : 100	Weak clot	Prevented	Prevented
1 : 1000	Very enhanced	Enhanced	Very enhanced
1 : 2,000	Very enhanced	Enhanced	Enhanced
1 : 4,000	Very enhanced	Enhanced	Enhanced
1 : 8,000	Very enhanced	Enhanced	Slightly enhanced
1 : 16,000	Enhanced	Slightly enhanced	No effect
1 : 32,000	Enhanced	Slightly enhanced	No effect
1 : 64,000	Slightly enhanced	No effect	No effect
1 : 128,000	Slightly enhanced	No effect	No effect
1 : 256,000	No effect	No effect	No effect

APPENDIX IV

ACTION ON FIBRINOGEN

	B. atrox	*B. nummifera*	*C. durissus*
1:10	No clotting	No clotting	No clotting
1:100	Weak clot in 2 min. Dissolution in 3 hrs.	No clotting	No clotting
1:1,000	Firm clot in 2 min. Dissolution in 5 hrs.	Weak clot in 2 min. Dissolution in 1 hr.	Weak clot in 4 min. Dissolution in 2 hrs.
1:10,000	Firm clot in 2 min. No dissolution.	Weak clot in 6 min. Partial dissolution in 17 hrs.	Weak clot in 6 min. Dissolution in 10 hrs.
1:100,000	Firm clot in 10 min. No dissolution.	Clot in 50 min. No dissolution.	Firm clot in 20 min. Partial dissolution after 17 hrs.
1:1,000,000	Firm clot in 2 hrs. No dissolution.	Clot after 12 hrs. No dissolution.	Firm clot after 1 hr. No dissolution.
1:10,000,000	Slight clotting after 14 hrs. No dissolution.	Slight clotting after 12 hrs. No dissolution.	Firm clot after 14 hrs. No dissolution.
1:100,000,000	Very slight or no clotting after 24 hrs.	No clotting after 24 hrs.	No clotting after 24 hrs.

APPENDIX V

PROTHROMBINASE ACTIVITY*

	B. atrox	B. nummifera	C. durissus
Presence of Calcium ions	Positive	Negative	Negative
Absence of calcium ions	Negative	Negative	Negative

* Venom dilutions ranged from 1 : 1,000 to 1 : 10,000.

APPENDIX VI

PROTEOLYTIC ACTIVITIES OTHER THAN TRYPSIN

Venom*	Rennin	Chymo-trypsin	Papain	Pepsin	Cathepsin
B. atrox	2+	2+	1+	-	-
B. nummifera	4+	4+	4+	-	-
C. durissus	4+	3+	2+	-	-

(*) Venom dilution in the reaction mixtures was 1:1,000.

APPENDIX VII

DIRECT HEMOLYSINS AND HEMAGGLUTININS

Venom*	Direct hemolysis	Agglutination
B. atrox	-	-
B. nummifera	2+	4+
B. nummifera (1: 800)	1+	-
B. picadoi	1+	3+
B. picadoi (1: 800)	-	-
B. nasuta	-	-
B. schlegeli	-	-
C. durissus	-	-
C. atrox	-	-
M. nigrocinctus	3+	-
N. naia	4+	-

* With the exceptions indicated in the table, final dilution of viper venoms was 80-fold. Concentration of M. nigrocinctus and N. naia venoms was 0.6 mg/ml.

APPENDIX VIII

COMPARATIVE PHOSPHATASE ACTIVITIES

AMP-ase[a]	NAD-ase[a]	ATP-ase[a]	DNA-ase[b]	RNA-ase[b]	Unspecific[c] phosphodi-esterase
		B. atrox			
66.4±26.7[d]	8.9±4.5	6.0±1.3	341±71	973±370	446±82.8
24.0-137[e]	4.0-22.5	1.3-9.4	120-650	352-2120	312-769
42[f]	42	42	40	39	40
		B. nummifera			
27.3±22.3	1.3±0.7	0.5±0.3	32.2±30.4	102±87	17±11.8
8.6-112	0.2-3.3	0.1-1.2	0-103.6	0-326	5.8-60
20	18	18	17	19	19
		B. picadoi			
54.8±18.6	2.7±0.9	4.0±0.8	285±57	629±163	318±20
31.4-79.0	1.7-4.1	2.5-5.4	186-400	266-844	269-339
9	10	11	10	10	10
		C. durissus			
69.3±38.5	6.6±2.7	4.6±2.4	222±75.7	135±62	227±45
30.0-147	3.6-12	2.4-9.8	103-378	55-230	158-304
10	10	10	12	9	10

[a] mM of phosphate released by 1 ml of venom per hour.
[b] µM of phosphate released by 1 ml of venom per hour.
[c] µM of p-nitrophenol released by 1 ml venom in 5 min.
[d] Avg. ± S.D. [e] Range [f] No. of samples.

APPENDIX IX

OTHER ENZYME ACTIVITIES

Trypsin[a]	L-amino acid dehydrogenase[b]	Phosphatidase A HD_{50}	Hyaluronidase VID_{50}[f]
		B. atrox	
254± 64[c]	29.4±5.4	1: 654,000	1: 1,030
140-400[d]	17.0-43.4	1:121,000-1:2,664,000	1:750-1:1,356
40[e]	42	41	12
		B. nummifera	
270± 33	25.7±4.2	1: 5,420	1: 1,592
231-370	18.9-31.4	1:4,448- 1:19,040	1:1,110-1:2,856
18	10	16	11
		C. durissus	
169±112	22.8±4.4	1: 4,450	1: 920
133-252	16.1-30.2	1:1,432-1:29,920	1:573 -1:1,578
10	10	12	10

[a] Trypsin activity is expressed in μM tyrosine released per milliliter of venom in 10 min.

[b] L-amino acid dehydrogenase is expressed in mM O_2/ml/hr.

[c] Avg. ± S.D. [d] Range [e] No. of samples [f] Pantlitschko and Kaiser (1951)

VITA

Jesús M. Jiménez-Porras was born on March 25, 1929 in the City of San José, Costa Rica. He attended the public schools of that city and graduated from "Liceo de Costa Rica". In 1948 he entered the University of Costa Rica, from where he graduated in 1953 with a Bachelor of Science degree (Microbiology and Clinical Chemistry). In 1950 he became a laboratory assistant in the Chemistry Department, University of Costa Rica. In 1952 he started teaching Organic Chemistry in several schools of the same University, where he continued working until 1958. At that time he was selected by the University of Costa Rica to hold a scholarship granted by INTERNATIONAL COOPERATION ADMINISTRATION (AGENCY FOR INTERNATIONAL DEVELOPMENT, United States Government), in order to do graduate work at Louisiana State University. While fulfilling the requirements for the Degree of Doctor of Philosophy in the Department of Biochemistry, Louisiana State University, he graduated with a Master of Science degree from the same department, in 1961. Immediately he returned to his country to head the Biochemistry Department of the new School of Medicine of the University of Costa Rica, where he has taught Biochemistry during the past two academic years. He established a serpentarium at the School of Medicine of Costa Rica in order to continue his

graduate studies on venoms of Costa Rican snakes, while being supervised by his major professors. At present he chairs a committee appointed by the Rector of the University of Costa Rica, to coordinate research in that University and to do preliminary studies on the possibilities of establishing a Graduate School in the University of Costa Rica. In January 1963 he returned to Louisiana State University to fulfill the residence requirement and to write his dissertation for the Doctor of Philosophy degree. In May, 1963, he was elected a member of the Louisiana State Chapter of the Society of the Sigma Xi.

EXAMINATION AND THESIS REPORT

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Major Field: BIOCHEMISTRY

Title of Thesis: COMPARATIVE BIOCHEMICAL STUDIES ON VENOMS OF SNAKES OF COSTA RICA

Approved:

Fred G. Brazda
Major Professor and Chairman

Max Goodwin
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Date of Examination:

MAY 15, 1963.